The causes and consequences of kin recognition in the cooperatively breeding cichlid *Neolamprologus pulcher*

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B. Sc. (Honours)

This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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June 2010

General abstract

Kin recognition allows individuals to assess their relatedness to conspecifics, thus they may then show kin discrimination and make informed choices as to with whom to associate and/or breed. Cooperatively breeding species, such as the cichlid *Neolamprologus pulcher*, are an excellent model system for investigating kin recognition, as they live in complex social groups, containing both kin and non-kin group members. Cooperation involves individuals helping to rear the offspring of the dominant pair. Helping is costly, but helpers may gain direct fitness benefits through living in a group, and if they aid relatives, they can also gain indirect fitness benefits through kin selection. Furthermore, by being able to recognise kin, individuals can also avoid inbreeding and the potentially deleterious consequences of it. Thus, *N. pulcher* are predicted to have good kin recognition abilities. In this thesis, I investigate kin recognition and its consequences for helping and mate choice in a captive population of *N. pulcher*. In chapter 2, I investigated the kin recognition capabilities of juvenile *N. pulcher* whilst controlling for familiarity. I found that *N. pulcher* preferred to associate with unfamiliar kin over unfamiliar non-kin. Kin recognition was via some form of phenotype matching, with chemical cues being more important than visual cues. Additionally, I found no discrimination between familiar and unfamiliar kin; thus, relatedness rather than familiarity was important in the association preferences of juvenile *N. pulcher*. Chapter 3 explored whether relatedness to the breeding pair, or differences in individual behavioural types affected the amount or type of helping shown by *N. pulcher*. Controlling for group size and helper relatedness, I found that the relatedness of the helpers to the breeders had no influence on the amount or type of help carried out. Thus, kin selected benefits alone cannot explain variation in helping behaviour in *N. pulcher*. The amount of territory maintenance carried out correlated with the amount of territory defence, thus, some individuals were consistently helpful. Individuals varied consistently in their aggressiveness, risk-responsiveness and activity levels, but these traits did not correlate with one another. More aggressive, risk-prone or more active helpers carried out more territory defence than submissive, risk-averse or inactive helpers. In contrast, the amount of territory maintenance carried out by helpers, was not correlated with the behavioural types. Thus, differences in behavioural types explained more

variation in helping behaviour in *N. pulcher* than relatedness. Since motivation to associate with kin might vary with age and individual state, in chapter 4, I investigated whether *N. pulcher* avoided kin when sexually mature, and examined the fitness consequences of inbreeding. In standard two-way choice tests, I found that whilst male *N. pulcher* showed no preferences for associating with sisters over female non-kin, female *N. pulcher* preferred to associate with brothers over male non-kin. However, when given the opportunity to breed, latency to breed and hatching success did not differ between brother-sister pairs and unrelated pairs. Thus, in *N. pulcher* inbreeding is not actively avoided and does not appear to be detrimental to fitness. I suggest that sex-biased dispersal and regular breeder replacement on territories may minimise the occurrences of inbreeding in the wild and that inbreeding may be opportunistic, rather than a strategic decision. The final theme of my thesis investigated the effect of phenotypic traits on mate choice. In *N. pulcher* (chapter 4) I found that the size of an individual's facial stripe, which varies between individuals, played no role in mate association preferences. I then investigated male mate choice for female body size in the non-cooperatively breeding green swordtail, *Xiphophorus hellerii*. In chapter 5, I found that males showed preferences for large over small females when presented only with visual cues, but not with only chemical cues. However, as the size differential between the large and small female increased, males showed preferences for the larger female based on chemical cues. So, male *X. hellerii* prefer larger females, which are predicted to be more fecund and hence, bring them greater fitness returns. In conclusion, my study has shown that *N. pulcher* can recognise kin, but the ability to do this does not compel individuals to show kin directed cooperation, or inbreeding avoidance. Instead, factors such as an individual's behavioural type have more influence on decisions to help, and inbreeding does not appear to be detrimental to fitness. Overall, this project shows that under the conditions we tested, kin selection alone does not drive the social interactions in *N. pulcher* groups. Further, it highlights the need to consider multiple factors affecting an individual's fitness, in order to fully understand why different species show a propensity to recognise and discriminate between kin and non-kin.

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Acknowledgements

First and foremost, I wish to thank my two supervisors Kate Arnold and Barbara Mable for their direction and support over the last few years. I have learned more than I could ever have imagined from them both and am ever grateful for all their help and encouragement. Although, I think I will always struggle with effects and affects, and I may never be sure where to put that comma!
Thanks also to Professor Michael Taborsky and Dr Dik Heg for supplying the stars of the show, the fish, as well as sharing their wealth of knowledge. Special thanks also to Dr Roger Schürch for his friendly advice and willingness to share his fish breeding expertise. Thanks also to Niall Gauld for help with data collection and for some great photos. The Biotechnology and Biological Sciences Research Council funded this study and I am grateful to them for giving me this opportunity to develop and hone my skills on such an interesting project.

Sincere thanks to everyone who helped to feed, clean and generally keep my fish and hence me happy over the past three years, particularly Graham Law, John Laurie, Helen Ablitt, Karen McLachlan and Graham Adams. I am massively grateful to all the other staff of the Graham Kerr Building, who keep the place running smoothly and are always on hand to help locate random pieces of equipment, print off bizarre pictures for birthday cards, supply essential keys for the wine and biscuit cupboard and to sign for parcels from e-bay! Namely, thank you to our amazing secretaries; Lorna and Florence, our excellent technicians; Pat, Kate, Irene, and Alistair and the wonderful janitors past and present; Davie, Alan and George.

Humungous chocolate-filled thanks to Aileen Adam for maintaining my sugar levels and providing me with first class training in the lab. Thanks also to Graeme Ruxton, for always offing up his amazing statistical mind for me to pick at, as well as supplying much needed advice and sweets! Gratitude also to Britt Heidinger, who's door was always open to questions.

To my wonderful office mates, my undying gratitude, you made the many long hours bearable and kept me sane. Steve Larcombe - I will always treasure our sing-alongs, your penchant for puns and that effortless ability you have to

brighten anyone's day! Katherine Herborn – for the many cups of tea and tears and tantrums (from both sides), I don't think I have ever giggled as much as I have done with you. Lindsay Henderson – your love of reggae and scat (Ski ba do bop de bo – meow!) and for putting up with my crazy cat. Leoni De Wert – I can't thank you enough for your support in these final few months, putting up with my mainly incoherent ramblings, sending me for massages, and generally keeping me from going insane without ever complaining once. You are an angel!

To the rest of the team GK; Andy 'Superted' Tedder, you made me laugh when things were bad and helped with the 'genetic stuff' when things were even worse – I heart you! Gus 'my ginger fox' Cameron, Gus B.C. (Before Coffee) is no use to man or beast, but fuel him up and he is good to go foxhunting or squid impersonating! Flavie 'emu' Vial, you will never fail to make me laugh without even meaning it! Not forgetting the 'old' school, Nick 'rosé' Beevers, Clare 'the wild dog' Marsden, Liz 'birthday fishes' Masden, and the ever elusive Will Miles. The 'new' school Vic Paterson, Eliza Leat, Zara Gladman, Gemma Jennings, Tim Burton, Donald Reid, Rob Critchlow and Julissa Tapia. And the 'retired' Chris Donaldson, Patrick Walsh, Tony Robertson, Peter Heobe, Tom Pike, Sunny Townsend, Chris Foote and Davina Hill – the fun times doing clockword, playing trivial pursuit and hula-ing in the sunshine will never be forgotten.

I couldn't have carried out this work without the love and support of my Aunty Joan, Uncle Gary, cousins Kayleigh and Callum, my brother Darryl, and finally my wee Gran and Grampa, who never really got what this ecology malarkey was all about, but supported me all the same. All my love and thanks to some amazing friends who stuck by me even when I was being antisocial and emotional, and provided me with cake, tea and love - Leanne, Pauline and Fiona, you are all amazing! And last, but by no means least, all my love and thanks to James, for his endless patience and support, putting up with the tears, tantrums and long hours and generally getting me through the craziness without a word of complaint. I heart you most. Thank you x

Candidate's Declaration

I declare that the work recorded in this thesis is entirely my own and is of my own composition. Niall Gauld aided in collecting data for chapter 4. No part of this thesis has been submitted for another degree.

Chapter 2 has been published in the Journal of Animal Behaviour; Le Vin, A. L., Mable, B. K. & Arnold, K. E. 2010. Kin recognition via phenotype matching in a cooperatively breeding cichlid, *Neolamprologus pulcher*. Animal Behaviour, 79, 1109-1114.

Appendix II has been published in Molecular Ecology Resources; Le Vin, A. L., Adam, A., Tedder, A., Arnold, K. E. & Mable, B. K. 2010. Validation of swabs as a non-destructive and relatively non-invasive DNA sampling method in fish. Molecular Ecology Resources, no. doi: 10.1111/j.1755-0998.2010.02909.x

Chapter 5 has previously been submitted for publication and is currently in revision. Chapter 3 is in collaboration with co-authors in Switzerland, who provided valuable comments on the manuscript and it is currently submitted to Behavioural Ecology and Sociobiology.

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June 2010

Chapter 1: General introduction

1.1 Primer

> "If it could be proved that any part of the structure of any one species had been formed for the exclusive good of another species, it would annihilate my theory, for such could not have been produced through natural selection." Darwin (1859)

Cooperation amongst species is an intriguing behaviour, as it apparently goes against Darwin's theory of evolution (1859) and the notion that individuals should be selfish and aim to maximise their own fitness. Yet, cooperation exists not only within species but even between them. For example: vampire bats, *Desmodus rotundus,* will share food, by regurgitating blood, for other individuals in their roost that have been unable to feed (Wilkinson, 1984); and ants of the species, *Pseudomyrmex ferruginea*, have a mutual relationship with the acacia, *Acacia cornigera,* whereby the ants receive food and shelter from the acacia in return for attacking other insects and plants invading it (Janzen, 1966).

Perhaps even more intriguing is the evolution of cooperatively breeding species, whereby individuals will forgo their own breeding in order to aid others. Hamilton (1964a; 1964b) proposed that cooperation may be driven by kin selection and the indirect fitness benefits that related helpers gain through helping. However, as unrelated as well as related individuals may cooperate, kin selection can not fully explain cooperative breeding, and other theories and studies challenge its importance (Clutton-Brock, 2002; Cockburn, 1998; Emlen, 1992; Griffin & West, 2002; 2003). Therefore, studies into cooperatively breeding species need to consider alternative reasons as to why individuals show altruistic behaviours via helping.

In this thesis, I address the kin recognition capabilities of the cooperatively breeding African cichlid, *Neolamprologus pulcher* and investigate whether kin recognition and selection influences the amount of cooperation they show towards other individuals. The thesis also considers other explanations for individual variation in cooperative tendency, specifically the effects of

individual behavioural types on helping effort. Furthermore, I explore whether kin recognition changes with the age and thus sexual maturity of an individual. The final theme of my thesis concerns decisions during mate choice; how sexually mature fish respond to individuals varying in relatedness and/or phenotype and the consequences of inbreeding. Also I explore mate choice decisions in a non-cooperatively breeding fish species, the green swordtail *Xiphophorus hellerii*.

1.2 Cooperative breeding

1.2.1 The evolution of cooperative breeding

Cooperative breeding can be broadly defined as when more than two individuals help to rear a single brood or litter. Cooperative breeding is a relatively rare behaviour (Emlen, 1992; Stacey & Koenig, 1990; Arnold & Owens, 1998) and the majority of studies focusing on it have been on birds (for reviews see; Brown, 1987; Koenig & Dickinson, 2004; Stacey & Koenig, 1990). However, examples of individuals aiding others to breed can also be found in mammals (Clutton-Brock *et al.*, 1998; Eberle & Kappeler, 2006), social insects (Field et al., 1999; 2006) and in fish (Heg & Bachar, 2006; Taborsky & Limberger, 1981). Cooperative breeding is a complex behaviour. In fact, in birds, Cockburn (2004) defines nine different types of mating systems. These range from true monogamy with helpers, where a monogamous pair breeds and is aided by others who do not gain sneak matings, to egalitarian polyandry, where groups of males are associated with a single female and aid in rearing offspring even though paternity is randomly distributed between them. Most cooperatively breeding species are characterised by having a hierarchical system, whereby a dominant breeding pair monopolises the majority, if not all, of the breeding, and subordinate individuals help (Griffin & West, 2003; Vehrencamp, 1983). Helping can take many forms; incubating/maintaining eggs, alloparental care of young, defence against predators and territory maintenance (Brown, 1987; Taborsky, 1984). Thus, cooperative breeding begs the question of why helpers would not disperse and breed and instead choose to help.

There are three main hypotheses as to why individuals choose not to disperse and breed independently: the ecological constraints hypothesis, the life history hypothesis and the benefits of philopatry. The ecological constraints hypothesis proposes that individuals are in some way constrained by their ecology, such as a specialised feeding habitat, low chance of finding a mate, or some high risk associated with leaving the natal territory, which then leads them to delay dispersal and breed cooperatively (Emlen, 1982; Koenig et al., 1992; Hatchwell & Komdeur, 2000; Arnold & Owens, 1999). Ecological constraints can lead to habitats becoming saturated with breeders. In the acorn woodpecker, *Melanerpes formicivorus,* a lack of suitable breeding territories and trees in which to store acorns, has led to habitat saturation (Stacey, 1979; Stacey & Ligon, 1987). This then leaves individuals with the option of either being philopatric, and perhaps helping, or dispersing and becoming a floater in the hope of gaining a territory when it becomes free (Koenig *et al.*, 1992). In the Seychelles warbler *Acrocephalus sechellensis*, ecological constraints on the amount of available breeding territories have led to individuals breeding cooperatively. Studies introducing individuals to unpopulated islands found that initially *A. sechellensis* breed independently, but individuals started to help on their natal territories as the good quality territories were filled up (Komdeur, 1992; Komdeur *et al.*, 1995). However, ecological constraints do not always lead to cooperation, as some species that are philopatric do not show helping behaviour (Veltman, 1989; Ekman et al., 1999). Therefore, ecological constraints may go some way to explain why some species do not readily disperse but it does not explain completely the evolution of cooperation.

The life history hypothesis has been suggested as another explanation for the evolution of cooperation (Hatchwell & Komdeur, 2000; Russell, 1989; Arnold & Owens, 1998; 1999). Here, a life history trait such as a low adult mortality rate leads to habitats becoming saturated and hence, there is a lack of breeding opportunities for new breeders. Thus, individuals that are unable to breed independently may help others in order to gain some fitness benefits. Whilst the ecological constraints hypothesis predicts that breeding is limited by the scarcity of an unusual resource that a species requires, the life history hypothesis suggests that it is due to a slow turn over of territories that habitats become saturated. However, the two hypotheses together may go further in explaining

why some species are cooperative, whilst others are not (Arnold & Owens, 1998; Hatchwell & Komdeur, 2000). However, there are always exceptions, such as the long tailed tit *Aegithalos caudate,* which often breeds cooperatively, but is short lived and does not retain its territory year round (Hatchwell & Russell, 1996). Thus, life history and ecological constraints theories still cannot fully explain cooperative behaviour in all species.

Finally, the benefits of philopatry, instead of focusing on costs and limitations, suggests that there are advantages to staying at home (Stacey & Ligon, 1991). Individuals remaining on their natal territory will have knowledge of its quality, the resources available and good foraging sites within it. Further, through living at home, individuals may be able to gain valuable skills, such as foraging experience (Heinsohn, 1991) or practice in raising young (Salo & French, 1989; Stone et al., 2010) which could increase their future reproductive success. Thus, the benefits gained through being philopatric may prompt individuals to also behave cooperatively within their social group. However, all three hypothesis, ecological constraints, life history and benefits of philopatry, are still heavily interlinked. Further, as cooperatively breeding species show huge diversity in their mating systems and behaviours (Cockburn, 2004), it would seem unlikely that a single reason could explain the evolution of cooperative breeding (Arnold & Owens, 1998; 1999; Cockburn, 1998; Hatchwell & Komdeur, 2000; Koenig et al., 1992).

1.3 Kin recognition and selection

1.3.1 Kin recognition mechanisms

If individuals wish to select relatives to help in order to gain indirect fitness benefits, then being able to recognise kin would be advantageous. However, it is important to point out that kin recognition and kin discrimination are two separate things; kin recognition is an internal, unobservable process, whilst kin discrimination can be seen as a difference in behaviour towards kin and non-kin (Waldman, 1988). Furthermore, a lack of kin discrimination in experiments does not necessarily mean that individuals cannot recognise kin; it may just be that they choose not to associate with or avoid them. Whether an individual chooses

to associate with kin may also depend on the individual's physiological state. When sexually mature, individuals may avoid kin to prevent inbreeding (Arnold, 2000). Therefore, studies into kin recognition can often be problematic, in that a result of no kin discrimination does not necessarily mean that no kin recognition exists. However, many studies have found species where individuals can recognise and discriminate between kin and non-kin, including fish (Arnold, 2000; Olsen, 1989; Griffiths, 2003), birds (Komdeur, 1994; Russell & Hatchwell, 2001), mammals (Mateo & Johnston, 2000; Mateo & Leslie, 2003), insects (Flores-Prado & Niemeyer, 2010; Page & Breed, 1987) and even plants (Dudley & File, 2007).

So, how do individuals recognise their relatedness to others in order to show kin selection? The ability of an individual to recognise kin may be innate, with a genetic component providing information about kinship, or it may be learned. Waldman (1987) suggested that kin recognition is composed of a series of events where an individual must give a signal, for example an odour or phenotype that is genetically determined, which must then be detected by another individual who then compares it to a template. If there is sufficient matching with the template then recognition will occur. Recognition of kin, however, is now widely thought to be a learned process (Hepper & Cleland, 1998), as it is unlikely that a single gene codes for all elements of a complex process like recognition. Individuals may learn to recognise kin in two ways. An individual may become familiar with other individuals it is raised with. Thus, they only recognise these familiar individuals as kin. Alternatively, an individual may learn some phenotypic cues of either the individuals with which it is raised (non self-referent phenotype matching), or itself (self-referent phenotype matching), and create a template with which to compare other unfamiliar individuals to assess relatedness (Holmes & Sherman, 1982; Lacy & Sherman, 1983; Blaustein, 1983). With phenotype matching, unlike kin recognition via familiarity, individuals can assess their relatedness to unfamiliar as well as familiar individuals. However, errors may occur when individuals use either familiarity or non self-referent phenotype matching in kin recognition. Nest mates may not actually be siblings or parents, for instance, in species where sperm can be stored, multiple mate copulations occur, or groups of young are raised communally (Hauber & Sherman, 2001). Some previous studies have failed to account for familiarity

between individuals so have found recognition based only on association, rather than true kin recognition (for review see Griffiths, 2003; and Ward & Hart, 2003). Hence, self-referent phenotype matching is the most failsafe method of truly recognising kin. However, it is difficult to experimentally distinguish self-referent from non-self referent phenotype matching. To achieve this, individuals need to either be individually marked and cross-fostered into an unrelated brood, or raised isolation from cues of other kin. Due to difficulties in cross-fostering and/or marking the young of some species, or the ethics of raising social species in isolation, self-referent phenotype matching has so far been demonstrated experimentally in a few species (Holmes & Sherman, 1982; Mateo & Johnston, 2000; Neff & Sherman, 2005). Therefore, it is important in studies of kin recognition to control for effects of familiarity and to be careful in interpreting results pertaining to the mechanisms of kin recognition.

However, it is important to note that preferences for associating with certain individuals may also be influenced by ecological factors like nutritional state, not just familiarity. Hungry three-spined sticklebacks, *Gasterosteus aculeatus,* have been found to prefer to shoal with unfamiliar non-kin over familiar non-kin, whilst satiated fish showed the opposite trend (Frommen et al., 2007a). As sticklebacks have been found to identify kin based on familiarity (Frommen et al., 2007b), it may be that when they are hungry they wish to reduce competition for food between potential kin by associating with unfamiliar individuals who they may not be related to. In addition to this, *G. aculeatus* prefer to associate with unfamiliar individuals that had experienced the same diet or environment treatment to themselves compared to familiar individuals from different diet or environment treatments (Ward et al., 2004) and these association preferences can develop in less that 24 hours (Ward et al., 2005). Furthermore, these preferences can be changed in as little as 3 hours if an individual is exposed to a different environment (Ward et al., 2007). This may be adaptive as associating with an individual from a similar environment may give additional information on that habitat, or could lead to increased competition for resources. In the Argentine ant, *Linepithema humile,* it is not adaptive as changing the diet of nestmates results in a breakdown of recognition and increased aggression (Liang & Silverman, 2000). Preferences for familiar, unfamiliar, kin or non-kin can therefore be context dependent and this has been

found in several species (Olsen et al., 2003; Pfennig 1990; Ward et al., 2004, 2005, 2007). Hence, in experiments looking at recognition the conditions that individuals are raised in needs to be taken into account, as it may be that preferences for individuals are based on context rather than recognition of familiars or kin.

Studies into kin recognition also often fail to tease apart whether chemical and/or visual cues are important for the recognition of kin or only investigate one potential recognition cue (Flores-Prado & Niemeyer, 2010; Komdeur, 1994; Mateo & Johnston, 2000; Mateo & Leslie, 2003; Olsen, 1989; Russell & Hatchwell, 2001; although note exceptions in Arnold, 2000; Plath et al., 2006). By differentiating between the importance of different cues, studies can further investigate how, and not just if, individuals can recognise kin. For instance, work suggests that genes of the major histocompatibility complex (MHC) influences an individual's odour (Singh et al., 1990). Genes of the MHC are highly polymorphic and encode glycoproteins that are involved in the recognition of self and non-self antigens in the immune system in vertebrates. Related individuals are likely to share alleles at the MHC, and studies have found that they may play a role in the recognition of kin (for reviews see Brown & Eklund, 1994; Penn & Potts, 1999; and studies in; Olsen et al., 1998; 2002; Rajakaruna et al., 2006). Other studies looking at polymorphic genetic markers, such as major urinary proteins (MUP's), have also found that they may be used for individual and kin recognition in mice (Hurst, 2009). However, whether MUP's play a role in chemical cues and kin recognition in fish has yet to be tested, and there is some evidence to suggest that the genes encoding MUP's may be specific to placental mammals (Logan et al., 2008).

Visual cues may also be used in kin recognition. Studies have found that individuals can use visual stimulus to recognise familiar individuals (Balshine-Earn & Lotem, 1998; Bergmüller et al., 2005a; Frostman & Sherman, 2004; Tibbetts, 2002). However, most studies that investigate visual cues in kin recognition use them in combination with chemical cues and don't tease apart their effects (Frommen et al., 2007b; Hain & Neff, 2007). Nevertheless, in an aquatic environment, chemical cues may be more easily detected over long distances and are not obscured by murky water. Hence, chemical cues may be more reliable indicator of kinship.

1.3.2 Why cooperate and help?

Helpers in cooperatively breeding groups carry out a range of helping behaviours that benefit the breeders, increasing their reproductive success, survival rates, clutch sizes and reducing their workloads (Balshine *et al.*, 2001; Emlen, 1992; Taborsky, 1984). However, being helpful comes at a cost to the helpers. In meerkats, *Suricata suricatta,* babysitting pups can cause subordinate helpers to lose on average 1.3%, but up to 11%, of their body weight (Clutton-Brock et al., 1998). Young white-winged choughs, *Corcirax melanorhamphos,* have also been found to lose body mass in proportion with the amount of incubation they contribute to (Heinsohn & Cockburn, 1994). Further, helpers may suffer costs through lost mating opportunities, reduced growth and energy expended while helping (Grantner & Taborsky, 1998; Taborsky, 1984). Therefore, for cooperative breeding to evolve, helpers must receive benefits to outweigh these costs. These benefits may be gained directly by increasing the survival and/or the reproductive success of the helper, or indirectly, via kin selection.

Individuals may gain direct fitness benefits that increase their survival, such as; protection from predators and larger conspecifics, by either the dilution effect or defence by larger group members (Hamilton, 1964b) or increased survival (Heg *et al.*, 2005). Alternatively, they may receive benefits that increase their reproductive success, such as; inheritance of a breeding territory (Woolfenden & Fitzpatrick, 1978), gaining parental experience (Salo & French, 1989; Stone et al., 2010) or gaining sneak matings with a breeder (Dierkes et al., 1999). Direct fitness benefits can be substantial. For example, in spotted hyenas, *Crocuta crocuta,* relatedness in cooperative groups is low; therefore, the direct fitness benefits gained through cooperation, such as gaining and maintaining access to food, must be sufficiently high to prevent independent breeding (Van Horn et al., 2004). Individuals can also gain direct benefits through group augmentation, which is the assumption that individuals will survive or reproduce better in larger groups, so it is beneficial to recruit more individuals into a group (Kokko et al., 2001). Kokko et al's (2001) model shows that the benefits gained through group augmentation are great enough to explain the costs of helping, even if some individuals cheat and reduce their helping effort. Alternatively, individuals may have to 'pay to stay' to be tolerated on a territory within a group (Gaston,

1978). Here, individuals help as a form of rent payment to be tolerated by the breeders. Work on superb fairy wrens, *Malurus cyaneus,* has found evidence of 'pay to stay', with helpers that were temporarily removed from the territory and hence, prevented from helping, being punished on their return for their defection from helping (Mulder & Langmore, 1993). Therefore, the direct fitness benefits gained by helpers, both related and unrelated to the breeders, can be considerable.

Helpers in many systems are related to the offspring that they are caring for (Griffin & West, 2002), so will share genes with these individuals, which will be passed on in subsequent generations, increasing the helper's fitness. Therefore, there has been a strong emphasis on the role of kin selection (Hamilton, 1964b) in the evolution of cooperative breeding. Hamilton's rule states that for helping to occur, **rb > c**, where **r** is the coefficient of relatedness, or the probability that the helper and recipient share a gene, **b** is the benefit gained by the recipient, and these multiplied together must be greater than **c,** which is the cost to the helper. Therefore, the theory makes two assumptions: that helpers are closely related to the breeders they are helping, and that they are improving the fitness of these breeders. Hence, by Hamilton's rule, it would be in an individual's interest to adjust their levels of helping depending upon their relatedness to the breeders, and this has been found in several studies (Clarke, 1984; Reyer, 1984; Komdeur, 1994; Wright et al., 2010; Russell & Hatchwell, 2001). However, the importance of kin selection in driving helping behaviour is questioned by studies that have found that the degree of relatedness does not always predict helping levels (Clutton-Brock et al., 2001; Wright et al., 1999; Canestrari et al., 2005). Furthermore, evidence that unrelated helpers may help more than related helpers, also questions how important indirect fitness benefits are in the evolution of cooperative breeding. For instance, subordinate male white-browed scrubwrens, *Sericornis frontalis,* were more likely to help if the female breeder they were aiding was unrelated to them, as they may also gain paternity in the clutch (Magrath & Whittingham, 1997). Therefore, although kin selection would predict that helping should be directed towards relatives, the direct fitness benefits gained through group living may be more than sufficient to drive individuals to help regardless of relatedness (Griffin & West, 2002; Clutton-Brock, 2002). However, if relatedness between the helpers and breeders is

unknown, it is difficult to distinguish if helpers are cooperating for mainly direct or indirect fitness gains. Hence, studies investigating helping in cooperative breeders must take into account the relatedness of helpers, and what effects these may have on helping effort.

1.4 Variation in individual levels of helping

As covered previously, the ability to recognise kin may encourage kin selection and cooperation between individuals. However, it is likely that factors other than kin selected benefits may influence how much help an individual is willing to carry out within its group. Variable factors may include the age or state of an individual that will determine whether they can pay the costs of helping. Other predictors of helping may be more permanent. Individuals have been found to show within-individual consistency and between-individual differences in behaviours, known as individual behavioural types (Sih et al., 2004a; Bell, 2007). The five most common axes of behavioural variation investigated between individuals are: exploration (fast – slow explorers) (Verbeek et al., 1994), aggression (aggressive – submissive/passive) (Riechert & Hedrick, 1993; Huntingford, 1976), risk responsiveness (bold – shy, risk prone – risk averse or neophobic – neophilic) (Wilson & Godin, 2009), activity (active – inactive) (Biro et al., 2009) and sociality (social – antisocial) (Cote & Clobert, 2007). So called behavioural syndromes, (Sih et al., 2004a; Realé et al., 2007), arise when different behavioural types are found to correlate over different contexts. For example, in three-spined stickleback, *Gasterosteus aculeatus,* Huntingford (1976) found a positive relationship between aggression to conspecifics and boldness towards predators. Components of behavioural syndromes are thought to be heritable. Work on the poeciliid fish *Brachyraphis episcope* found heritability of boldness, with offspring of bold parents being bold, and those of shy parents being relatively shy (Brown et al., 2007). Further, behavioural types can also be shaped by early life experiences such as hormones, nutrition or maternal effects (see review by Sih et al., 2004b; and study by Arnold et al., 2007). Thus, individuals may be predisposed to exhibit behaviours, that may in turn affect their fitness, such as foraging (Herborn et al., 2010) or showing territory maintenance or defence (Bergmüller & Taborsky, 2007; Schürch & Heg, 2010). So behavioural types and syndromes may reveal ecologically significant

variation between individuals that could affect fitness related traits (Realé et al., 2007; Sih et al., 2004a), such as helping effort.

More recent work has found that behavioural types and their associated behavioural syndromes are not always consistent in different populations of the same species. Bell (2005) investigated two populations of three-spined sticklebacks, and found a behavioural syndrome linking aggression, boldness and activity in only one of the populations. Bell (2005) suggested that the differences in behaviour seen between the populations may be shaped by predation pressures. This was confirmed by work by Dingemanse et al (2007), who found that populations of sticklebacks that lived in large ponds with predators exhibited the behavioural syndrome found by Bell (2005), whilst populations living in small ponds with no predators did not. Thus, it is likely that there will be extensive differences between populations in the suite of behaviours they exhibit, depending upon which pressures are acting upon them and hence what behaviours would optimise their fitness in their particular environment. Therefore, it is important to consider that behavioural syndromes may change between populations and to consider the multiple factors that may be influencing them.

In addition to the behavioural traits discussed above, levels of cooperative behaviour shown by individuals have been found to be variable (reviewed in Bergmüller et al., 2010). This has been found in different taxa including fish (Schürch and Heg, 2010a; Schürch and Heg, 2010b), birds (Komdeur & Edelaar, 2001), mammals (O'Riain et al., 1996) and insects (Hughes and Boomsma, 2008). If cooperative behaviours are repeatable they could also be classed as a behavioural type. Consistency in helping behaviours shown by an individual may help to maintain group stability or it could bring benefits to either an individual or the group as a whole. For example, individuals may attain a territory or mate, whilst the group may increase its reproductive success or improve its foraging ability (reviewed in Bergmüller et al., 2010). However, cooperative groups are open to cheating with some individuals offering little or no help but still gaining the fitness benefits of the group, leading to the Prisoner's Dilemma of whether to cooperate and help or to defect (Axelrod and Hamilton, 1981). Therefore, it is intriguing as to how repeatable variation in cooperation could evolve as individuals receive the greatest payoff by defecting from helping. However,

models have shown that variation in behaviour in a group that is maintained by extrinsic factors, such as mutation and immigration, can lead to stable high levels of cooperation (McNamara et al., 2004). Further, McNamara et al. (2009) have also predicted through models that if individuals can monitor the cooperative behaviours of others, which is costly to the individual to do, then this social awareness and trust of others' cooperative behaviours results in stable consistent individual differences in behaviour. It is also possible that individuals are consistently helpful in order to reduce or avoid punishment from other group members (Balshine-Earn et al., 1998, Bergmüller & Taborsky 2005 and reviewed in Bergmüller et al., 2010). Alternatively, it may be costly, or individuals may be limited in the amount of behavioural plasticity they can show (Dewitt et al., 1998), hence they are consistent in their cooperative behaviours. However, to date few studies have investigated the consistency of cooperative behaviours (although see English et al., 2010; Schürch & Heg, 2010), thus further work is needed.

1.5 Kin recognition and inbreeding avoidance

Kin recognition can be beneficial if individuals wish to select kin to cooperate with, as described previously. However, individuals may not always wish to associate with and/or help kin, so, preferences may change with the age and condition of an individual. A major advantage of kin recognition may be to avoid kin when sexually mature to prevent the risk of inbreeding; in zebrafish, *Danio rerio*, sexually immature individuals recognise and prefer to associate with unfamiliar kin over unfamiliar non-kin via phenotype matching. However, when sexually mature, this preference changes and instead they avoid kin (Gerlach & Lysiak, 2006), indicating that they are avoiding inbreeding.

Inbreeding at an individual level is defined as mating with a relative, or in the case of self-fertilising species, mating with oneself. At the population level, inbreeding is defined as mating between individuals that are more related to each other, than to another individual in the population chosen at random. As related individuals share at least one recent common ancestor, they also share some genes, which are described as being identical by descent. Inbreeding, therefore, reduces heterozygosity, depleting genetic variation within

populations, making them less able to respond to changing environments (Keller et al., 1994). An excess of homozygotes, due to breeding between genetically similar individuals, can lead to the expression of deleterious alleles in individuals. This has been found to reduce fitness related traits such as offspring survival, fertility, competitive ability and body size (reviewed in Keller & Waller, 2002). Inbreeding is a particular problem where populations are small, fragmented or where there is little dispersal, and can eventually lead to extinction (Frankham, 1998). Furthermore, in cooperatively living species, where groups often contain related individuals (Dierkes et al., 2005; Russell & Hatchwell, 2001; Stacey & Koenig, 1990), inbreeding may occur if individuals can not recognise kin and avoid them as mates. Therefore, regardless of whether species are cooperative or not, we may expect that mechanisms of kin recognition may be more developed to avoid inbreeding in those species that encounter kin more often than in species that seldom encounter kin. However, many species that don't live in kin structured cooperative groups still interact with kin regularly. For example, species that are philopatric and remain close to home, socially living species and lekking species can often encounter kin (reviewed in Hatchwell, 2010), so could still benefit from having the ability to recognise and avoid kin as mates.

Whilst inbreeding is generally thought to be detrimental, there is mounting evidence that inbreeding may not always be bad (Kokko & Ots, 2006). Research has found that outbreeding with individuals that are very genetically different can in fact be unfavourable, with studies finding reduced offspring size, survival and hatching success (Peer & Taborsky, 2005; Sagvik et al., 2005; Waser & Price, 1989). Inbreeding can be advantageous, and increase an individual's inclusive fitness, when the benefits gained are greater than the costs ensued by inbreeding, and/or if alternative opportunities to mate are not lost (Kokko & Ots, 2006). Further, it can help to prevent the loss of genes adapted to that population or environment (for review see Edmands, 2002). Naturally high levels of inbreeding have been found in some species, such as the dwarf mongoose, *Helogale parvula* (Keane et al., 1996). In this species, individuals do little to avoid inbreeding, with little dispersal by both males and females from their natal group, and mating at random with respect to relatedness. Furthermore, there appears to be no detrimental effect of inbreeding to either adult or

offspring survival (Keane et al., 1996). If breeding opportunities are limited, the cost of not breeding at all may be greater than the cost of inbreeding. In species that breed only once annually, where locating mates is infrequent or breeding territories are limited, trading off the costs of inbreeding may be the best option to increase an individual's fitness. So, inbreeding may be advantageous in some species, detrimental in others and/or may reflect the need to trade off its costs with the cost of foregoing breeding.

1.6 Mate choice

In addition to assessing relatedness, other factors are important when selecting who to breed with. Mate choice can be defined as behaviour shown by one sex, that leads to them being more likely to mate with certain members of the opposite sex, than with others (Halliday, 1983). As females invest more in producing eggs, they are generally the choosier sex, whilst males normally compete for the female's attention. The theory of sexual selection (Darwin, 1871) predicts that males should maximise their fitness by reproducing with as many females as possible, whilst females should choose males of high quality, through which they will gain fitness benefits (Trivers, 1972). However, mate choice can be exercised by both sexes, and it has been argued that males should be choosy in situations where females differ in quality (e.g. fecundity, size or parental abilities), when males have access to a selection of females to mate with, and/or where sperm reserves are depleted after one mating, thus reducing the chances of fertilizing subsequent females (Andersson, 1994). So, males too may try to choose a mate of the highest quality in order to maximise their fitness return.

Individuals may exhibit mate choice to gain direct fitness benefits that increase their fitness or fecundity (Kirkpatrick & Ryan, 1991; Reynolds & Gross, 1990). They may choose a mate who provides them with a good territory (Searcy, 1979), nuptial gifts (Reinhold, 1999) or who shows good parental care (Forsgren, 1997). Further, phenotypic traits, such as body size may predict direct fitness benefits from mate choice. In fish, body size in females reflects fecundity, with larger females producing more, and/or larger eggs (Bagenal & Braum, 1968; Cheong et al., 1984; Kraak & Bakker, 1998; Plath et al., 2006). Consequently,

males choosing larger females as mates, may gain additional fitness benefits over those mating with smaller females. Alternatively, individuals may chose mates who will pass on good genes that should improve the fitness or attractiveness of their offspring (Andersson, 1994; Trivers, 1972). Elaborate secondary sexual ornaments, such as the tail feathers of peacocks and the swords of swordtails, have been proposed to serve as markers of individual quality, as only the best quality individuals can afford to exhibit them (Zahavi, 1975). Indeed, many sexually selected phenotypic traits have been found to be costly to the bearer. Consequently, only the best quality individuals can 'afford' to display elaborate ornaments. In male sticklebacks, *Gasterosteus aculeatus,* the intensity of red colouration negatively correlated with numbers of parasites, (Milinski & Bakker, 1990), and in green swordtails, *Xiphophorus hellerii,* males with longer swords incurred greater swimming costs, but showed enhanced escape abilities over shorter sworded males (Royle et al., 2006). Thus, phenotypic traits can serve as honest signals of mate quality. Certain phenotypic traits may also act as 'badges of status', that indicate an individual's social status within a group, which may also be used in mate choice. In house sparrows, *Passer domesticus*, males have black throat patches that signal dominance status, with males with larger patches being dominant (Møller, 1987). These, males also hold larger territories with more breeding sites within them, and females show preferences for mating with these males (Møller, 1987). Therefore, badges of status can serve as a signal of an individual's ability to maintain its social ranking, and hence are measure of its fitness. However, it is interesting to note that preferences for these 'badges of status' can change between populations. In a closed population of house sparrows, with little emigration or immigration, females preferred males with smaller patches, and these males had higher breeding success (Griffith et al., 1999). Again, this emphasises the fact that there can be differences in behaviours and preferences shown between populations.

1.7 *Neolamprologus pulcher*

This thesis focuses on *Neolamprologus pulcher,* a cooperatively breeding cichlid species endemic to Lake Tanganyika in Africa. *N. pulcher* lives on rocky substrate at depths ranging from 3 to 45 meters (Taborsky, 1984), where it

maintains a territory and breeds in rocky crevices. *N. pulcher* grows to around a maximum size of 65mm standard length (SL), and is known to be sexually mature by around 35mm SL and approximately 8-9 months old (Dierkes et al., 1999; Taborsky, 1985). Social groups of *N. pulcher* are governed by a size dominance hierarchy consisting of a large dominant breeding male and female, and between 1 – 14 helpers who vary in size, sex and relatedness (Balshine et al., 2001; Taborsky & Limberger, 1981). Some dominant males, however, are polygynous (Limberger, 1983), having several breeding females on nearby territories. *N. pulcher* is an ideal species for carrying out captive experiments. It is small, has large clutch sizes and reaches sexual maturity relatively quickly. Further, *N. pulcher* exhibit the full range of cooperative behaviours in captivity as they do in the wild.

1.7.1 Costs and benefits to helpers

Helpers aid the breeders by carrying out a range of tasks, including cleaning and fanning eggs, removing sand and snails from the breeding shelters, and defending the territory and other group members against predators and intruding conspecifics (Balshine-Earn & Lotem, 1998; Taborsky, 1984; Taborsky & Limberger, 1981). As discussed previously, helping is costly to helpers, and beneficial to recipients. Specifically, in *N. pulcher*, helpers have increased energy expenditure when helping compared to normal swimming (Grantner & Taborsky, 1998), have reduced growth rates (Taborsky, 1984) and lose out on mating opportunities (Taborsky & Limberger, 1981). Although helpers are not punished by breeders for defecting from helping they have been found to be punished by other helpers for absconding, and can even be evicted from the territory (Balshine-Earn et al., 1998). Furthermore, helpers temporarily prevented from helping increased their helping effort on their return to the territory to apparently appease the breeders and other helpers for their absence and lack of help (Bergmüller and Taborsky, 2005). Studies have also found that *N. pulcher* helpers increased their helping effort when there was a threat that their position in the territory may be taken over by another conspecific (Bruintjes & Taborsky, 2008), with larger helpers who are more likely to gain a breeding spot showing a greater increase in helping than smaller helpers. Breeders with helpers on the other hand reap the benefits of reduced workloads

(Balshine et al., 2001) and increased future reproductive success (Taborsky, 1984). Furthermore, breeders with large numbers of helpers produce larger clutches and have higher offspring survival (Brouwer et al., 2005; Taborsky, 1984) than those with few helpers. Of course helpers must also gain benefits for helping to evolve. Group living brings direct fitness benefits to *N. pulcher* through increased survival due to having access to a shelter and protection by larger group members (Taborsky, 1984). Female helpers can inherit a breeding spot on their natal territory (Stiver et al., 2006), whilst male helpers may sire up to 22.9% of a clutch by parasitizing reproduction (Dierkes et al., 1999; 2008; Heg et al., 2006). Furthermore, if helpers are related to the breeding pair they may gain additional kin selected benefits (Hamilton, 1963; 1964b). *N. pulcher* exhibit natural helping behaviours in captivity, furthermore, they can be stimulated to help experimentally using standardized helping experiments. For example, breeding shelters can be artificially filled in with sand to prompt digging behaviour by helpers, and a conspecific intruder can be introduced to a territory and helpers will show defensive helping behaviours. Thus, they are an ideal species in which to investigate whether indirect or direct benefits are driving helping effort.

1.7.2 Relatedness in groups

Relatedness between helpers and breeders in *N. pulcher* has been found to vary widely within groups. Breeders are replaced over time, with males being replaced more frequently than females (Stiver et al., 2004). This, in combination with females often inheriting territories, means that larger and therefore older individuals have been found to be distantly, if at all related to the breeders, in particular to the breeding male (Dierkes et al., 2005). Further, Stiver et al (2005) found that helpers were on average only related to the breeding female at the level of first cousins ($r = 0.125$) and unrelated to the breeding male. Therefore, smaller, younger helpers who are more related to the breeders may be more likely to help due to kin selected benefits, whilst larger, older helpers may help for direct fitness gains, such as parasitizing breeding (Brouwer et al., 2005; Dierkes et al., 2005; Stiver et al., 2005). This mixture of relatedness within groups of *N. pulcher,* make them an ideal species in which to investigate kin recognition, as individuals may adjust their helping effort dependent upon

how related they are to the individuals they are helping. Thus, *N. pulcher* would be expected to have finely tuned kin recognition abilities.

1.7.3 Recognition of conspecifics

Previous work on *N. pulcher* has found that adult males recognise, and show less aggression towards familiar versus unfamiliar male neighbours, based only on visual cues (Frostman & Sherman, 2004), the so called 'dear enemy' effect (Fisher, 1954). Neighbouring territory holders are hypothesised to pose relatively less of a threat to an individual than an unfamiliar conspecific. This is because the neighbour already has a territory whilst the stranger may not, hence, costly aggressive acts towards them can be reduced compared to those against strangers (Temeles, 1994). Alternatively, it may be that familiarity can reduce the likelihood of a role mistake in a territory dispute, as individuals will have already interacted and established their roles in the past (Ydenberg et al. 1988). In addition to males showing more aggression to unfamiliar conspecific intruders, *N. pulcher* breeders have been found to show less aggression towards familiar versus unfamiliar helpers (Hert, 1985) based on both visual and chemical cues. These individuals should pose little threat to the breeder in terms of territory take over, so increased aggression towards them is likely to be to establish their place in the dominance hierarchy. Finally, helpers have been found to show preferences for associating with their own social group as opposed to an unfamiliar group (Jordan et al., 2010), again using both chemical and visual cues. Consequently, *N. pulcher* can recognise and discriminate between familiar and unfamiliar individuals. Hence, it would seem likely that *N. pulcher* should have finely tuned kin recognition abilities that would enable particularly smaller, younger helpers, to receive kin-selected benefits through helping relatives. However, whether *N. pulcher* are capable of recognising kin, whilst controlling for familiarity, has yet to be experimentally tested under controlled conditions.

1.7.4 Effects of relatedness on helping effort

Studies on *N. pulcher* have found mixed results concerning the relatedness between group members, and how much helping effort helpers show. In the

field, Stiver et al (2005) found that overall relatedness to the breeding pair did not influence the amount of helping individuals carried out. However, when relatedness scores to the male and female breeders were looked at separately, it was found that helpers related to the breeding female and helpers unrelated to the breeding male, carried out the most territory defence. In contrast, relatedness scores to either the male or female breeder had no influence on the amount of territory maintenance (digging and removing sand and debris from the breeding shelter), carried out (Stiver et al., 2005). However, the same study also found that in the laboratory, helpers unrelated to the breeding pair showed more territory defence and maintenance than helpers related to the breeding pair (Stiver et al., 2005). However, these results were obtained by only observing natural levels of helping in both the wild and captive groups. Thus, no manipulations to standardise the level of helping effort between groups were carried out. Consequently, there was likely to be substantial variation between groups in the amount of helping required by individuals, which could have had major influences on the results Stiver et al (2005) found. Thus, it remains to be tested in *N. pulcher*, whether relatedness has real effects on helping effort. In order to fully test this, laboratory manipulations using groups of *N. pulcher* that are standardized for size, familiarity and relatedness would need to established, and then regulated helping challenges carried out, to assess the effects of relatedness on helping effort. As with other species (Dierkes et al., 2005; Magrath & Whittingham, 1997; Van Horn et al., 2004), it may be that kin selection alone cannot fully explain individual variation in helping behaviour, and other explanations need to be investigated.

1.7.5 Individual variation in personality traits and helping

In addition to kinship, individual differences in personality traits have been found to play a role in the amount and type of helping behaviour shown by individual *N. pulcher*, although the findings between studies are not consistent. One study, investigating sexually immature individuals, found that more explorative male and female *N. pulcher,* defended their territory more, than less explorative individuals (Bergmüller & Taborsky, 2007). Further, this study found that territory maintenance was negatively correlated with helper aggression, when in the presence of breeders (Bergmüller & Taborsky, 2007). In

another study, levels of aggression, boldness and exploration were found to be correlated in sexually immature females, and mature males and females, but not immature males (Schürch & Heg, 2010). Conversely, Schürch and Heg (2010) found that, in both juveniles and adults, more explorative females carried out more territory maintenance than less explorative females, and not more territory defence, as Bergmüller and Taborsky (2007) had found. Therefore, there seems to be differences between populations in their behavioural syndromes. Further, neither of these studies took into account the relatedness of the helpers to the breeders which may also have affected helping effort. A better approach might be to control for relatedness between group members, and then explore whether differences in individual personality traits affects not only the type, but the amount of helping an individual shows.

1.7.6 Adult kin recognition and inbreeding avoidance

Kin recognition may be used as a tool to prevent inbreeding in sexually mature individuals. As discussed previously, it is expected that a socially living species like *N. pulcher* should have finely tuned kin recognition abilities. However, the potential for inbreeding can also be reduced in wild populations by sex-biased dispersal. This aids inbreeding avoidance even if individuals can not recognise kin, as only one sex moves away from the natal territory to breed, thus eliminating the chance of breeding with kin. In *N. pulcher*, males are generally the dispersing sex (Stiver et al., 2004; 2007), so the chance of breeding with relatives should be reduced. However, field studies in *N. pulcher* found that individuals breed at random, neither actively avoiding nor seeking out related individuals as breeding partners (Stiver et al., 2008). However, as this study investigated the degree of allele sharing between individuals, it could not assess whether shared alleles were identical by descent. Therefore, individuals that bred may just have shared alleles by chance, rather than actually being relatives. Nonetheless, even with sex-biased dispersal in this species, individuals still breed with genetically similar individuals. This lack of inbreeding avoidance in adult *N. pulcher* may be due to them not being able to recognise kin, to avoid them as potential mates, or inbreeding may be advantageous and therefore, not avoided. However, whether adult *N. pulcher* can recognise and choose to avoid kin as potential mates, has not been tested. Further, the willingness of *N.*

pulcher to breed with kin, and the possible negative effects inbreeding may have on breeding success, has yet to be investigated.

1.8 Male mate choice in the Green swordtail, *Xiphophorus hellerii*

In addition to investigating mating decisions in the cooperatively breeding cichlid *Neolamprologus pulcher*, my thesis also investigated male mate choice in the non-cooperatively breeding poeciliid fish species, the green swordtail, *Xiphophorus hellerii*. *X. hellerii* is found in streams and rivers throughout Central and South America. It is a small, sexually dimorphic, freshwater fish species belonging to the live bearing family Poeciliidae. Females grow throughout life, whilst males only grow until sexually maturity, when they stop growing in body size and develop a 'sword' (Basolo, 1990), a colourful, elongated extension of the male's caudal fin. In swordtails, female mate choice has been extensively studied, and experiments have shown that females prefer longer-sworded (Basolo, 1990), larger-bodied males (Basolo, 1998; Wong et al., 2005) and well fed males (Wong et al., 2005). Male mate choice in swordtails on the other hand, has been less well studied. A study by Benson (2007) found that male *X. hellerii* courted females with artificially enlarged gravid patches more often than females without enlarged gravid patches. The gravid patch is a dark spot found on the lateral aspect of a female's abdomen, and is bigger when a female is carrying larger or more eggs. Accordingly, the size of the brood patch should indicate a female's fecundity. And as larger females should be more fecund, it may be expected that male *X. hellerii* should prefer larger over smaller females as has been found in other fish species (Cote & Clobert, 2007; Herdman et al., 2004; Sargent et al., 1986; Werner et al., 2003). However, whether male *X. hellerii* show preferences for larger females, or if larger females produce more or larger offspring, has yet to be examined.

1.9 Aims of thesis

The main aim of my thesis was to investigate kin recognition and its consequences on the social behaviour of *N. pulcher*, using carefully controlled experiments and manipulations. In **Chapter 2**, I investigate whether *N. pulcher*

can recognise kin over non-kin when sexually immature, whilst controlling for the effects of familiarity. Further, in this chapter I examine whether chemical or visual cues are more important in kin recognition in this species. In **Chapter 3,** I assess whether the relatedness of helpers to the breeders influences the amount and/or the type of helping carried out. In addition to relatedness, I also investigate the effects of individual behavioural types on helping effort shown by *N. pulcher*. **Chapters 4** and **5** examine mate choices in *N. pulcher*, as well as in the non-cooperatively breeding live-bearing poeciliid fish, the green swordtail *Xiphophorus hellerii*. In **Chapter 4,** I focus on inbreeding avoidance, specifically asking whether *N. pulcher* continue to show preferences for opposite sex kin over non-kin, when sexually mature. I also assess the influence of phenotypic traits on adult mate preference. Further, I investigate the propensity of individuals to inbreed and examine the consequences of inbreeding on fitness. Finally, in **Chapter 5** I explore male mate choice in *X. hellerii* and examine whether males prefer large, presumably more fecund females, over small females, and whether chemical and/or visual cues are more important in mate choice.

Chapter 2: Kin recognition via phenotype matching in a cooperatively breeding cichlid, *Neolamprologus pulcher*

2.1 Abstract

Cooperatively breeding groups are often made up of a mixture of related and unrelated individuals. In such groups, being able to identify and differentially cooperate with relatives can bring indirect fitness benefits to helpers. I investigated the kin recognition abilities of the cooperatively breeding African cichlid *Neolamprologus pulcher,* while controlling for familiarity between individuals. When given a choice of associating with unfamiliar kin or unfamiliar non-kin, juvenile *N. pulcher* spent significantly longer associating with kin. Although both chemical and visual cues were required to stimulate the fish, chemical cues were more important than visual cues in kin recognition in this species. As all stimulus fish were reared separately from the focal fish, I can also conclude that *N. pulcher* used phenotype matching rather than familiarity to assess relatedness to other individuals. Furthermore, when given the choice of associating with familiar over unfamiliar kin, *N. pulcher* showed no significant preference. Hence, relatedness rather than familiarity appears to be more important in the association preferences of *N. pulcher*. This is advantageous, particularly if familiar individuals within the cooperatively breeding group are not necessarily relatives. In highly social species such as *N. pulcher,* being able to recognize kin can bring fitness advantages through kin selection and inbreeding avoidance.

2.2 Introduction

Cooperative breeding can be broadly defined as when more than two individuals help to rear a single brood or litter. Cooperative breeding is a relatively rare behaviour (Emlen, 1992; Stacey & Koenig, 1990), but is found in a range of taxa including birds (Stacey & Koenig, 1990), mammals (Eberle & Kappeler, 2006), invertebrates (Field et al., 1999) and fish (Taborsky & Limberger, 1981). Helping can take the form of incubating/maintaining eggs, provisioning young, predator defence, nest cleaning and territory maintenance (Brown, 1987; Taborsky, 1984). As helpers in many systems are related to the offspring that they are caring for (Griffin & West, 2002), there has been a strong emphasis on the role of kin selection (Hamilton, 1964b) in the evolution of cooperative breeding. Individuals that help kin can gain indirect fitness benefits through helping to raise offspring with which they will share genes. Moreover, sexually mature individuals can avoid the deleterious consequences of inbreeding (Pusey & Wolf, 1996), if they are able to recognize and avoid kin as mates. However, cooperatively breeding groups often also contain unrelated individuals and, consequently, there must be other fitness benefits gained through being cooperative. Group living can confer direct fitness advantages regardless of relatedness, such as reduced predation because of the dilution effect (Hamilton, 1964b), inheritance of a breeding territory (Woolfenden & Fitzpatrick, 1978), sneak matings (Dierkes et al., 1999) and increased survival (Heg et al., 2005). Thus, species that are able to recognize and choose to help kin will receive additional indirect fitness benefits through kin selection as well as the direct benefits gained through group living.

Being able to assess relatedness between individuals is important for kin selection. The ability of an individual to recognize kin is likely to be a learned process (Hepper & Cleland, 1998). Individuals can learn to recognize kin in two ways. First, individuals may interact with and become familiar with the traits of kin they have had prior association with; thus, they learn to recognize specific individuals with whom they are familiar. Alternatively, an individual may learn phenotypic cues either of the other individuals it is reared with (non self-referent phenotype matching), or from itself (self-referent phenotype matching), and make a template from this with which to compare others

(Holmes & Sherman, 1982; Lacy & Sherman, 1983). The drawbacks of using familiarity as a recognition method is that only familiar individuals can be recognized, whereas by phenotype matching both familiar and unfamiliar individuals can be assessed and their relatedness determined. There is also room for error where individuals use familiarity or non self-referent phenotype matching to learn relatedness. For example, in species where multiple mate copulations occur, where sperm can be stored or where groups raise young communally, apparent relatives may be half siblings or completely unrelated (Hauber & Sherman, 2001). Therefore, self-referent phenotype matching is the most failsafe way of recognizing kin. Kin recognition has been found in a range of species (Arnold, 2000; Bateson, 1982; Brown et al., 1993; Mateo & Johnston, 2000; Neff & Sherman, 2005). However, previous studies have sometimes failed to account for familiarity between siblings, or have found recognition based only on association (Reviewed in Griffiths, 2003; Ward & Hart, 2003) rather than showing true kin recognition.

Neolamprologus pulcher is a cooperatively breeding cichlid endemic to Lake Tanganyika in East Africa. These fish live in social groups consisting of a dominant breeding pair and up to 14 related and unrelated helpers (Balshine et al., 2001). The dominance hierarchy among group members depends on size. Helpers clean and fan eggs in the breeding shelter and help to defend the territory and other group members against predators and intruding conspecifics (Balshine-Earn et al., 1998; Taborsky, 1984; Taborsky & Limberger, 1981). Helpers in this species incur costs such as reduced growth (Taborsky, 1984), lost mating opportunities (Taborsky & Limberger, 1981) and higher energy expenditure when showing helping behaviours compared with routine swimming (Grantner & Taborsky, 1998). However, being allowed to live in a group has direct fitness benefits for *N. pulcher* helpers. Helpers have increased survival because they have access to a shelter and protection from larger group members (Taborsky, 1984), and male helpers may parasitize the reproduction of the breeders (Dierkes et al., 1999). Furthermore, female helpers may go on to inherit a breeding spot on their territory (Stiver et al., 2006). Related helpers may also receive additional indirect benefits through aiding kin. There is already evidence that *N. pulcher* is able to recognize familiar individuals using only visual cues, based on males showing more aggression towards unfamiliar than

familiar male neighbours (Frostman & Sherman, 2004). Additionally, breeders using both chemical and visual cues recognize and show less aggression towards familiar helpers than to other conspecifics (Hert, 1985) and helpers show preferences for associating with familiar over unfamiliar groups (Jordan et al., 2010). Therefore, it would seem likely that *N. pulcher* should have finely tuned kin recognition abilities, which would enable them to receive kin-selected benefits through helping relatives, but this has yet to be established.

The aim of my study was to determine whether *N. pulcher* can recognize kin over non-kin independent of familiarity. First, I investigate whether *N. pulcher* show any preferences for kin over non-kin, when familiarity with each group is controlled for, based on chemical and/or visual recognition cues. The second aim was to determine whether *N. pulcher* show any preferences for associating with familiar over unfamiliar kin, to evaluate whether familiarity with individuals is more important in deciding with whom to associate than overall relatedness. Fish used in the kin recognition trials were sexually immature, so preferences for associating with other individuals indicate social rather than sexual preferences. All fish used were known not to be inbred (see chapter 4) which could have otherwise confounded my results.

2.3 Methods

2.3.1 Breeding Design

In November 2006, adult *N. pulcher* were caught in Lake Tanganyika, in Zambia, by members of the Bern Diving Expedition, and transported to the University of Bern, Switzerland, by air, under licence from the Ministry of Agriculture and Co-operatives in Zambia. In March 2007, 68 adult *N. pulcher* were transported by air, from the University of Bern, to the University of Glasgow, under licence from the Scottish Executive Environment and Rural Affairs Department. Fish were transported in two insulated polystyrene crates (59 x 40 cm and 33 cm high), with each crate containing six thick plastic bags, stocked with four to six fish each. The fish were in transit for less than 12 h and water temperature was maintained above 20 °C throughout. During this period, fish were not fed as the addition of food would have degraded water quality. As the fish are normally fed

once every 24 h, this was not an unduly long period without food. During transit to Glasgow no mortality occurred. However, eight fish died in the subsequent 2 weeks after their arrival. The causes of these deaths were not obvious, but were not due to physical injury. The fish received came from two areas in the southern tip of the lake. One group of 32 adults were wild-caught fish, from Nkumbula Island, near Mpulungu. The other group of 36 adults were F1 generation fish, from wild-caught fish from Kasakalawe Bay, near Mpulungu, caught in 1996. Fish were kept within their group in mixed-sex tanks until breeding began. These holding tanks ranged in size from 50 to 250 litres and stocking densities ranged from 3 to 27 individuals. Tanks were provisioned with 1 - 1.5 cm of coral sand on the base, an air stone, foam filter, and several clear plastic tubes suspended at the top of the tank to act as refuges. The water temperature was kept in the range of 26.8 ± 1 °C, pH in the range 8 - 8.4 and a light regime of 13:11 h light:dark. Adult fish were fed once daily with either a commercial dry cichlid food, frozen bloodworm or *Daphnia*.

In early June 2007, I paired individuals with an opposite-sex partner from the same area of the lake as themselves. Fish were anaesthetised using a benzocaine solution and sexed by examining the genital papilla. A single male and female were then randomly paired and placed into a 140-litre breeding tank (80 x 40 cm and 50 cm high). Water parameters, lighting and feeding regimes were as described previously. Breeding shelters were provided and consisted of two pieces of plastic pipe cut in half and two terracotta flowerpot halves. Each of the shelters had the inside surface covered with a thin flexible plastic layer that was clipped in place. Females laid eggs on the sides of the shelters, and so the plastic layer with the eggs attached could be removed easily. Shelters were checked for eggs every morning and evening. When eggs were found they were left with the breeding pair for a further 24 h after which they were removed and the clutch counted. Each clutch was split into two to provide groups of individuals who were familiar with the siblings they had been reared with (familiar siblings) but were unfamiliar with the siblings reared separately (unfamiliar siblings). To achieve this, the two half clutches from each brood were transferred to two visually and chemically isolated 50-litre tanks (48 x 27 cm and 35 cm high), in which water parameters were the same as in the parental breeding tanks. When the fry started to feed independently, at around

10 days old, they were fed on a combination of crushed flaked food and frozen Cyclops or *Daphnia* twice a day. In total 35 pairs laid eggs but only 26 of these pairs produced offspring that survived in each of the two groups. These offspring were then used in the subsequent kin recognition experiments.

2.3.2 Experimental Design

I carried out two experiments between October 2007 and March 2008, each testing a single offspring from each of the pairs of breeders. These experiments investigated the preference of a focal fish for associating with one group of stimulus fish over another. The first experiment looked at preferences for kin over non-kin and the second investigated preferences for familiar over unfamiliar kin. Pilot studies (A. Le Vin, unpublished data) with juvenile *N. pulcher* found that when isolated from the visual cues of conspecifics and given only a chemical stimulus, individuals often remained immobile in one corner of the tank or made irregular darting movements. However, fish behaved normally, swimming freely around the tank and investigating the preference zones, when presented with visual cues alongside chemical cues. Furthermore, individuals showed more interest in groups of three conspecifics than in a single stimulus fish. Each experiment consisted of two tests: a matched-cues test and a mismatched-cues test, each of which consisted of two trials. In total, four trials (two matched cues and two mismatched cues) were run over 4 consecutive days for each experiment, in a randomized order, and using the same focal fish in each of the four trials. In the matched-cues test, the visual cues from the stimulus fish matched their chemical cues (chemical cues from A to X and B to Y, see Figure 2-1). In the second trial of the matched-cues test the sides on which the cues were presented were swapped to control for side biases. In the mismatched-cues test, the visual cues from the stimulus fish did not match their chemical cues. So, in one side of the tank the focal fish could view one stimulus group but simultaneously received the chemical cues of the other stimulus group and vice versa on the opposite side of the tank (chemical cues from A to Y and B to X, see Figure 2-1). Again, in the second trial of the mismatched-cues test I controlled for side bias by swapping the sides on which each stimulus was presented. From the mismatched-cues test I could disentangle whether chemical or visual cues may be more important for kin recognition in *N. pulcher*.

Between experiments, all of the fish used were returned to their original home tanks containing their familiar siblings. I did not mark fish for identification as this could have affected my experiments, which relied on visual cues for recognition. Each home tank generally contained several fish of a size suitable for testing, so it was unlikely, although not impossible, that the same individual was tested as the focal or stimulus fish in each of the two experiments. However, as there was a minimum of 59 days between experiments, I do not expect that this would systematically bias my results.

2.3.3 Experimental Protocol

An individual focal fish was introduced to a 5-litre experimental tank (32 x 17 cm and 19 cm high), 22 h prior to a trial to allow it to acclimate. Adjacent to the experimental tank were two stimulus fish tanks (17 x 10 cm and 19 cm high) each filled with 1.75 litres of water (see Figure 2-1). All water used in experiments was kept within the same parameters previously described in the breeding design. To provide water with chemical stimulus cues, three fish were introduced into each of the stimulus tanks at the same time as the focal fish. Filters were not included in either the experimental or stimulus tanks as they could have affected the chemical cues produced by the fish. Therefore, I checked water quality regularly to confirm that it remained within safe levels for the fish. The experimental and stimulus tanks were covered with card on three sides to prevent the fish being disturbed by my observations, and to prevent the stimulus fish from seeing each other. Removable card barriers were positioned between the experimental and stimulus tanks to prevent the focal fish from seeing the stimulus groups during the acclimation period. The experimental tank was divided into two regions: two ‘preference zones’ located adjacent to the front of each stimulus tank measured 13 cm long by 8 cm wide. Between each of these preference zones was a 6 cm ‘no preference zone’ and the rest of the tank was also a no preference zone (see Figure 2-1). Water was drawn from the stimulus tanks, through the pump and dripped into the middle of the preference zone through silicone tubing which I secured above the tank by slotting it into a piece of rubber tubing attached to the side of the experimental tank. Flow rate was set to 1.4 ml/min, which is sufficient to induce a reaction to a chemical stimulus (McLennan & Ryan, 1997). Flow rate was checked regularly

to ensure accuracy. Pilot studies with colour-dyed water showed that the water dripping from the pumps remained concentrated and mainly localized within the preference zones. To observe the fish during the trials from a distance without disturbing them, I placed a mirror above the tank.

At the start of each trial the pumps were started and the card barriers removed so that the focal fish could see the stimulus fish. The trial started when the focal fish was in the no preference zone so that the fish's preference was not biased if it started off in one of the preference zones. The fish was then observed for a 10 min period and the time spent in each of the preference zones recorded. The focal fish had to enter both preference zones in at least three of the four trials in the matched- and mismatched-cues tests for the experiment to be valid, which ensured that the fish took part in at least one of either the matched or mismatched trials. Furthermore, this helped to control for any side biases and made certain that the fish was swimming normally, investigating both preference zones within the tank in more than 50% of the trials. After each trial, the focal fish was removed from its tank and the experimental tank was cleaned thoroughly with 100% alcohol and rinsed with a powerful jet of water. This removed the chemical cues from the stimulus fish and therefore prevented the focal fish becoming overly familiar with them. The tank was then refilled and the focal fish reintroduced. All fish were then fed either crushed dry food or frozen *Daphnia* and left to feed. Approximately 30 min later, all fish were removed from their tanks and placed into temporary holding tanks while both the experimental and stimulus tanks were cleaned with 100% alcohol, rinsed with water and refilled with fresh water. This removed any traces of food which would have degraded water quality and may have otherwise affected the chemical cues. The fish were returned and given another 22 h acclimation period before the next trial commenced. This protocol was followed until the fish had completed all four trials. The same silicone tubing was used in the matched- and mismatched-cues tests, and care was taken to ensure that the same tubing was used for the same stimulus group over the four trials. Between trials the tubing was flushed with clean water and then allowed to empty to remove chemical traces from the previous trial. The tubing was then completely changed between experiments, so that fresh tubing was used for each different focal fish that was tested.

The sizes of the focal and stimulus fish were initially matched by eye before the experiment began and their standard lengths (SL) were measured after the 4 days of trials had ended, to avoid excessive handling prior to testing.

2.3.4 Kin versus Non-kin

For the kin versus non-kin experiment, a focal fish had the choice of associating with either a stimulus group of unfamiliar kin or unfamiliar non-kin. In this experiment, the experimental and stimulus fish did not differ significantly in either age (focal and kin stimulus fish: mean age = 103.05 ± 2.08 days; non-kin stimulus fish = 101.64 ± 2.40 days; Mann Whitney *U* test: $U = 238.5$, $N_1 = 22$, $N_2 = 66$, $P = 0.94$) or SL (focal fish: mean SL = 19.66 ± 0.40 mm; kin stimulus fish = 19.69 ± 0.35 mm; non-kin stimulus fish = 19.33 ± 0.37 mm; Kruskal Wallis test: $H_{21} = 0.33$, $P = 0.85$). A total of 22 individuals completed the four trials.

2.3.5 Familiar versus Unfamiliar Kin

Individuals were also tested for their preferences for a stimulus group of familiar kin or unfamiliar kin. As in the kin versus non-kin experiment, the focal and stimulus fish did not differ in age (focal and stimulus fish: mean ± S.E. = 165.83 ± 7.14 days) or SL (focal fish; mean ± S.E.= 27.42 ± 1.14 mm; familiar kin stimulus fish; mean ± S.E. = 25.43 ± 0.96 mm; unfamiliar kin stimulus fish; mean ± S.E. = 25.85 ± 1.03 mm; Kruskal Wallis test: $H_{17} = 4.43$, $P = 0.11$). In this test 18 individuals completed all four trials.

2.3.6 Data Analysis

All data were analysed using SPSS version 10.0.5 (SPSS Inc., Chicago, IL U.S.A.). Data were checked for normality and homogeneity of variance. For the kin versus non-kin experiment, paired t-tests were carried out to determine whether the focal fish spent longer with either kin or non-kin in the matched-cues test. To tease out which cue was most important in kin recognition, I compared the time spent with kin in the matched-cues test, where they had both chemical and visual cues from kin, with the time spent with either the chemical or the visual cues of kin during the mismatched-cues test. For the familiar versus unfamiliar kin experiment, a Wilcoxon signed-ranks test was carried out, as data were not

normally distributed, to establish whether the focal fish spent longer with familiar or unfamiliar kin in the matched-cues test. All tests were two tailed.

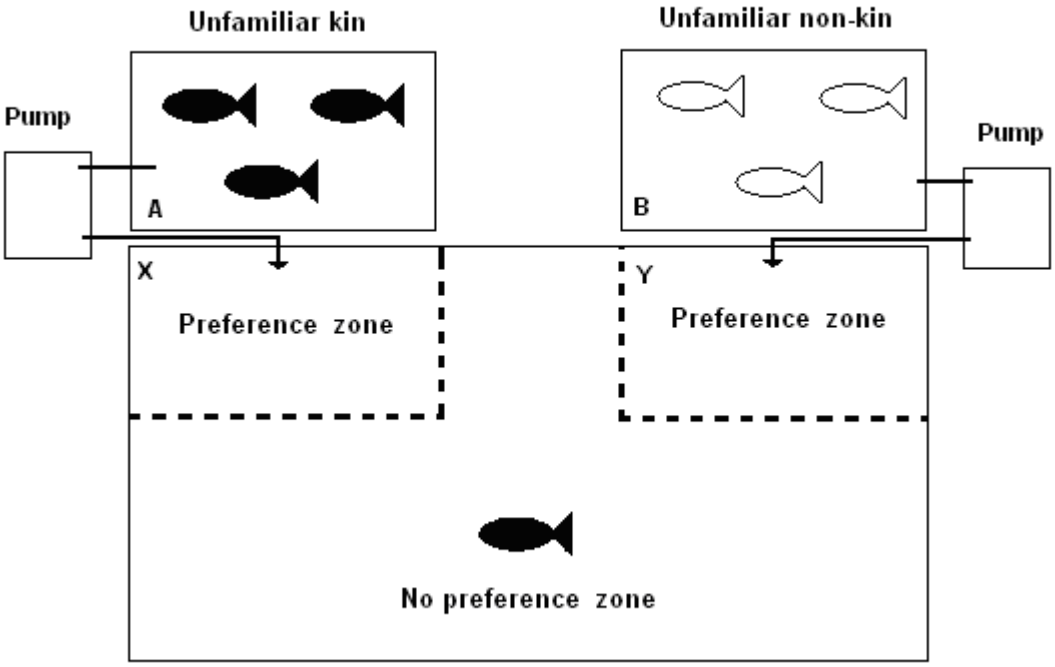

Figure 2-1. Schematic of experimental set up for kin recognition experiments. Dashed lines indicate the two preference zones in the experimental fish tank. In the matched cues test, the pumps carried stimulus water from tank A to preference zone X and from tank B to preference zone Y as shown. In the mismatched cues test the pumps carried stimulus water from tank A to preference zone Y and tank B to preference zone X. Diagram not to scale.

2.4 Results

2.4.1 Kin versus Non-kin

In the matched-cues test, *N. pulcher* spent significantly longer with kin than with non-kin (paired t-test; t_{21} = 2.15, P = 0.04; Figure 2-2). Therefore, *N. pulcher* preferred to associate with unfamiliar kin over unfamiliar non-kin, when presented with both chemical and visual cues. Individuals spent significantly longer with kin when chemical and visual cues were matched than when they had visual contact with kin in the mismatched-cues test (paired t-test; t_{21} = 2.55, P = 0.02; Figure 2-3a). However, I found no significant difference in the time spent with kin when visual and chemical cues matched and when *N. pulcher* had the chemical cues of kin in the mismatched-cues test (paired t-test; t_{21} = 0.86, P = 0.40; Figure 2-3b). Taken together these results suggest that in juvenile *N. pulcher,* chemical cues are more important for the recognition of unfamiliar kin, although visual cues still play a role in stimulating the fish to seek the proximity of conspecifics.

2.4.2 Familiar versus Unfamiliar Kin

Analysis of the matched-cues test found that *N. pulcher* showed no significant preference for spending time with either familiar or unfamiliar kin (Wilcoxon signed–ranks test; Z = -1.24, N = 18, P = 0.23; Figure 2-4). Therefore, *N. pulcher* do not prefer to associate with kin based on familiarity.

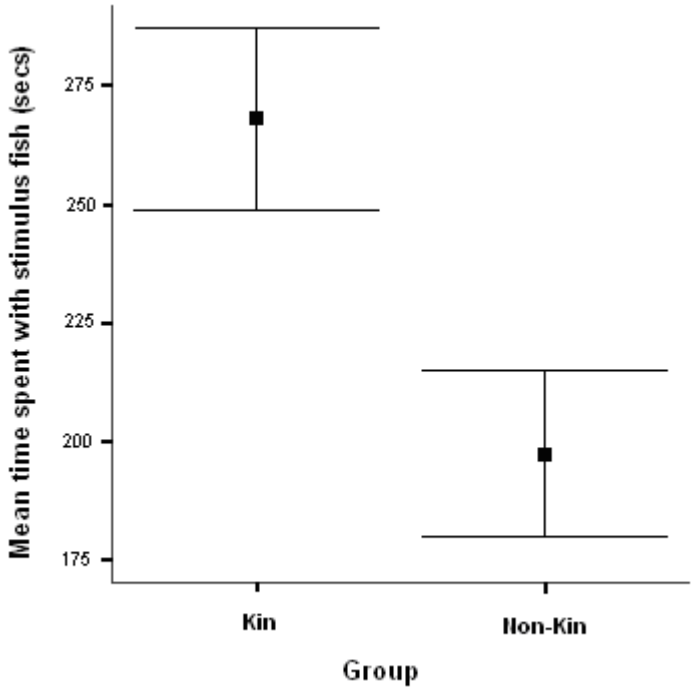

Figure 2-2. Kin versus non-kin experiment. Mean time spent with kin and non-kin groups in the matched cues experiment. Error bars show the mean ± S.E.

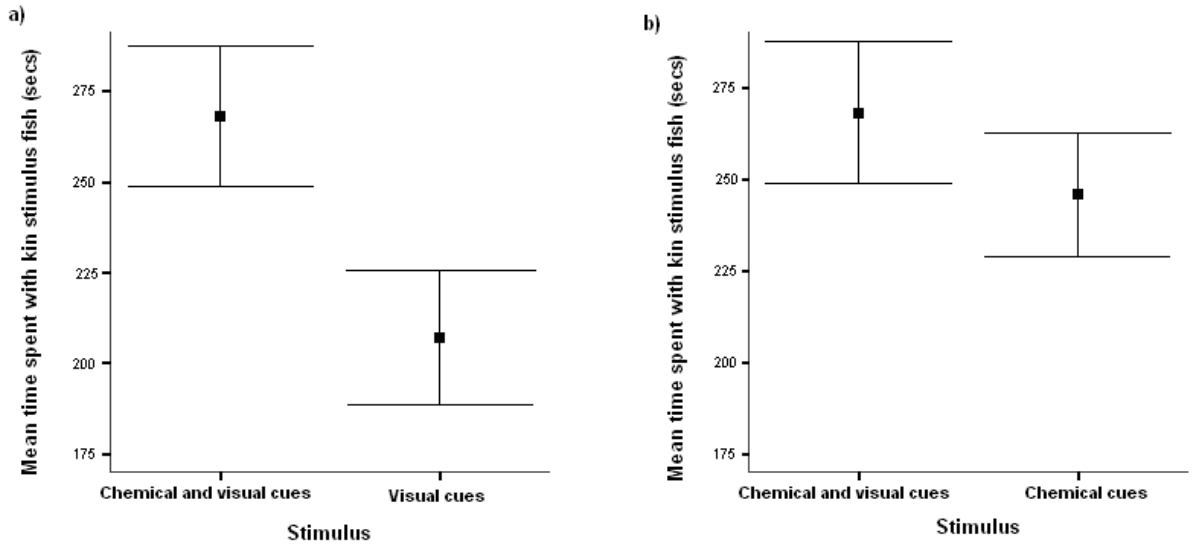

Figure 2-3. Kin versus non-kin experiment. Mean time spent with kin with either a) chemical and visual cues in the matched cues test compared with visual cues in the mismatched cues test, or b) chemical and visual cues in the matched cues test compared with chemical cues in the mismatched cues test. Error bars show the mean ± S.E.

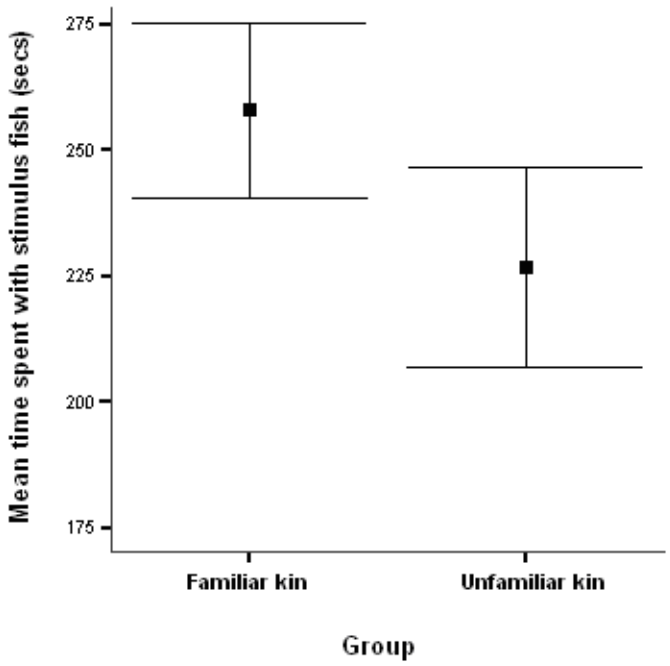

Figure 2-4. Familiar kin versus unfamiliar kin experiment. Mean time spent with familiar and unfamiliar kin groups in the matched cues test. Error bars show the mean ± S.E.

2.5 Discussion

I found evidence that *N. pulcher* can recognize and show preferences for associating with unfamiliar kin over unfamiliar non-kin using phenotype matching. Further to this, it seems that chemical cues are more important in kin recognition than visual cues, which seem to be mainly necessary to stimulate the fish to associate with its conspecifics. I also found that in *N. pulcher* familiarity does not affect preferences for kin, which may be advantageous as familiar individuals are not necessarily relatives. This result is contrary to previous work that found that helper *N. pulcher* preferred to associate with familiar over unfamiliar groups (Jordan et al., 2010). However, Jordan et al's (2010) study did not take into account relatedness between the focal fish and the groups. The familiar group was the focal fish's original social group, whereas the unfamiliar groups were collected from over 50 m away. Therefore, it was possible that the focal fish were related to the individuals within their group, which may have affected their association choices. Furthermore, differences in size and potentially in sexual maturity between the fish in my study and that of Jordan et al. (2010) may also account for differences in preference for familiar or unfamiliar groups. The fish I tested were all sexually immature and of a size where they would be helpers in a natural group situation. Thus, if individuals choose to help relatives, regardless of their familiarity to them, they can receive indirect fitness benefits via the young they help to raise. In other fish species, such as rainbow trout, *Oncorhynchus mykiss*, and brown trout, *Salmo trutta*, living with kin has also been found to reduce costly aggressive interactions and improve weight gain (Brown & Brown, 1993a; Olsen et al., 1996). Therefore, living with relatives potentially could confer further advantages to individuals. Further to these benefits, recognizing kin could also aid inbreeding avoidance when individuals become sexually mature. Evidence from studies of *N. pulcher* in the wild showed that individuals appear to breed randomly, neither actively avoiding nor preferring relatives as mates (Stiver et al., 2008). However, this may be because of a lack of breeding opportunities, and so individuals given the chance to breed may do so regardless of their relatedness to a mate. Thus, kin recognition in *N. pulcher* may not be used predominantly for inbreeding avoidance and the indirect benefits gained through kin selection may be more important.

My results suggest that chemical cues are required for kin recognition in *N. pulcher*, while visual cues seem to play a lesser role by stimulating the fish to seek the proximity of other fish. Ideally, I would have tested the fish giving them only visual and only chemical cues to tease out more clearly their relative importance in kin recognition. However, during pilot studies I found that fish behaved normally, by swimming freely around the tank, when they were provided with both chemical and visual cues simultaneously (A. Le Vin, unpublished data). Being highly sociable, group-living animals, *N. pulcher* seem to require both stimuli initially to evoke them to associate with the stimulus groups. Chemical cues have been found to be important in kin recognition and association preferences in other fish species. For example, fish of the genus *Xiphophorus* have been found to use chemical cues in mate choice (see chapter 5) and to recognize conspecifics (McLennan & Ryan, 1997; Wong et al., 2005), and bluegill sunfish, *Lepomis macrochirus,* and rainbow trout show recognition of unfamiliar kin using only chemical cues (Neff & Sherman, 2005; Olsen et al., 1998). Chemical cues may be a more reliable cue for kin recognition as they are likely to be detected at greater distances than visual cues, which could be obscured in turbid waters. The source of these chemical cues indicating kinship in this species is currently unknown, but may have a genetic basis in either genes of the major histocompatibility complex (MHC; Olsen et al., 1998) or major urinary proteins (MUPs; Hurst, 2009).

Visual cues were required by *N. pulcher* to stimulate them to associate with the focal fish, but are relatively unimportant for recognition of kin. Previous work in *N. pulcher* and sister species *N. brichardi* has found that adults can recognize familiar conspecifics using visual cues (Balshine-Earn & Lotem, 1998; Bergmüller et al., 2005a; Frostman & Sherman, 2004). Adult *N. pulcher* have distinctive facial stripes, and it has been suggested that they may aid visual recognition of familiar individuals (Duftner et al., 2007). However, these facial stripes take time to develop and were not present in the juveniles I used (A. Le Vin, personal observations), and in my study I found no preference for familiar over unfamiliar kin. Familiarity and visual cues may therefore be more important in interactions between unrelated adults.

As the experimental fish were unfamiliar with both the kin and the non-kin stimulus groups, I can conclude that *N. pulcher* must be using some form of

phenotype matching in their kin recognition. Whether *N. pulcher* are using self-referent phenotype matching, where they use their own phenotype as a template with which they compare other individuals, or are using a phenotype template of the siblings they were reared with is unknown. Previous work has found that extra-pair paternity occurs within groups of *N. pulcher,* with large male helpers accounting for up to 10.3% of the offspring in a clutch (Dierkes et al., 1999). Therefore, in order for individuals to infer their relatedness to others reliably, self-referent phenotype matching would be the best option for true kin recognition in this species. This could be assessed experimentally by either raising an individual on its own or cross-fostering one individual into another brood. However, currently this would be logistically difficult in a small fish species and unethical in a social species like *N. pulcher*.

A possible confounding effect in my study is the fact that my familiar and unfamiliar groups of kin were together during the egg phase for approximately 2 days. As eggs only take around 3 - 4 days to hatch, at 2 days, embryos could be well developed and it is possible that chemical transfer between eggs in a clutch in this short timeframe may aid recognition of otherwise unfamiliar individuals. However, in other fish species, familiarity takes some time to develop (Griffiths & Magurran, 1997), and cues of relatedness may not be produced until eggs have hatched (Neff & Gross, 2001). So it would seem unlikely that a short time spent together at the egg stage could have affected the results by individuals becoming familiar with one another during this phase.

In summary, I have shown that *N. pulcher* can recognize and choose to associate with unfamiliar kin over unfamiliar non-kin. Further to this, chemical cues are required for kin recognition to occur, with visual cues only being necessary to stimulate the fish to associate with its conspecifics. However, as to what the chemical cues are, and how they differ between related and unrelated individuals, is at present unknown. I can, however, conclude that *N. pulcher* shows true kin recognition, using some form of phenotype matching. As *N. pulcher* is a cooperatively breeding species with limited dispersal and groups that are made up of related and unrelated individuals, the ability to recognize kin could bring fitness advantages. If individuals are not limited in their breeding opportunities and can avoid inbreeding they can receive direct fitness benefits

via kin recognition. Furthermore, they can also increase their fitness indirectly if they chose to help raise the offspring of relatives.

Chapter 3: Effects of relatedness and behavioural type on helping effort in the cooperatively breeding cichlid, *Neolamprologus pulcher*

3.1 Abstract

Cooperatively breeding groups often contain helpers of varying levels of relatedness to the breeders. Individual helpers can vary hugely in the type and level of help they provide. Kin selection alone cannot be invoked to explain variation in helping for many cooperatively breeding species, but there have been few explicit tests of this under controlled conditions. Here, I investigated whether relatedness to the breeding pair, or consistent individual differences in behaviours between helpers, affected the amount or type of helping shown in the cooperatively breeding cichlid, *Neolamprologus pulcher*. I set up standardised social groups containing a breeding pair and one related and one unrelated helper, both unfamiliar to each other and to the breeding pair. Two forms of helping, territory maintenance and territory defence, were measured repeatedly under controlled conditions. I found that helping was variable between, but consistent within, individuals. Furthermore, helpers that carried out more maintenance also carried out more defence. Interestingly, I found that relatedness did not affect the amount, or type, of helping carried out by the helpers. Risk-taking, activity levels and aggressiveness differed between individuals but were consistent within individuals. These three behavioural types were not correlated with each other. I found that more aggressive, risk-prone or more active helpers carried out more territory defence than submissive, risk-averse or inactive helpers. In addition, there was a trend for risk-prone individuals to carry out more territory maintenance than risk-averse individuals. Overall, differences in individual behavioural types, rather than relatedness explained more variation in how much helping behaviour *N. pulcher* carried out. This study highlights the importance of considering multiple factors when investigating complex behaviours, such as helping.

3.2 Introduction

Helpers in cooperatively breeding groups carry out a range of helping behaviours, including provisioning young with food, predator defence, deterring conspecific intruders, nest maintenance and care of eggs (Emlen, 1992; Stacey & Koenig, 1990; Taborsky & Limberger, 1981). Individual helpers can vary in terms of who they help, and also in the type, and level, of help they provide. The presence of helpers within a group has been found to increase the reproductive success of the breeders, increase survival rates of both breeders and offspring, reduce workloads for the breeders and increase clutch size (Balshine et al., 2001; Brouwer et al., 2005; Emlen, 1992; Taborsky, 1984). Although helpers are generally beneficial to the breeders, it comes at a cost . Costs of helping include lost mating opportunities, energy expended helping and risk of injury defending the territory (Clutton-Brock et al., 1998; Heinsohn & Legge, 1999; Taborsky, 1984; Taborsky & Grantner, 1998). The benefits accrued by kin selection may offset some of these costs, as related helpers will have some of their genes passed on in the offspring they are helping to raise (Hamilton, 1964b). Indeed, some studies have found that individuals adjust their helping depending upon relatedness to the breeders (Clarke, 1984; Emlen & Wrege, 1988; Komdeur, 1994; Reyer, 1984; Wright et al., 2010). If helpers are only receiving kin selected benefits, then only related helpers should help. However, the importance of kin selection in driving helping behaviour is questioned by studies that have found that the degree of relatedness does not always influence helping levels (Canestrari et al., 2005; Clutton-Brock et al., 2001; Wright et al., 1999), and also by the presence of unrelated helpers in cooperative groups (Dierkes et al., 2005; Magrath & Whittingham, 1997; Van Horn et al., 2004). Therefore, direct fitness benefits gained through group living may be sufficient to drive individuals to help (Clutton-Brock, 2002; Griffin & West, 2002). Both related and unrelated helpers can accrue direct fitness benefits, which may include reduced predation risk, increased foraging abilities, the chance to inherit a territory or gain sneak matings with a breeder (Hamilton, 1964b; Heg et al., 2004; 2005; Dierkes et al., 1999; Taborsky, 1984; Woolfenden & Fitzpatrick, 1978). As related helpers receive both direct and kin-selected fitness benefits, they may be expected to help more than unrelated individuals. However, it has also been suggested that individuals may be charged ‘rent' in the form of helping in order to ‘pay to stay’

on the territory and be tolerated by the breeders (Gaston, 1978). In return, they gain the direct fitness benefits of group living. Both related and unrelated helpers may have to pay, although it has been hypothesised that unrelated helpers may be expected to help more than a related helper (Kokko et al., 2002), as they may be less likely to be tolerated by breeders than a relative. So, if kin selection yields few benefits because of low relatedness between helpers and beneficiaries, and/or the direct benefits of group living are more important than kin selected benefits, then we may expect all helpers to help equally regardless of relatedness. Similarly, if individuals must 'pay to stay', then all helpers regardless of relatedness may need to help. However, if 'charges' are altered according to the relatedness of helpers to breeders, then unrelated helpers may need to help more than related ones.

The type of helping carried out may also be influenced by the relatedness of a helper to the breeders it is aiding. Related helpers may be predicted to perform more costly or risky helping, such as territory defence against an intruder, in which an individual may suffer injuries or be killed through fighting in order to protect their family members and territory (Balshine et al., 2001). In contrast, territory maintenance, such as digging sand and debris out of shelters used for breeding and hiding from predators, should be relatively low risk, in terms of causing personal injury. Thus, unrelated helpers may be expected to focus on less risky behaviours. So, relatedness may predict the type, not simply the level, of helping performed by a helper.

Factors other than relatedness may also influence the amount and type of help an individual is willing or able to provide. The study of so called 'animal personality' is a relatively new subject area in evolutionary biology, and subsequently there is ongoing discussion in the literature as to definitions surrounding it (Gosling, 2001; Realé et al., 2007; Sih et al., 2004b). Therefore, I shall define the terms used commonly within the literature, and those that I wish to use in the context of this study. Faced with the same environment or behavioural stimuli, and measured under standardised captive conditions, individuals of the same species often show 'consistent individual differences' in behaviour, or 'behavioural types' (Bell, 2007; Sih et al., 2004a). Commonly measured axes of behaviour include: aggression (aggressive – submissive) (Riechert & Hedrick, 1993), activity (active – inactive) (Biro et al., 2009),

sociality (social – antisocial) (Cote & Clobert, 2007), exploration (fast – slow explorers) (Verbeek et al., 1994) and risk responsiveness (risk prone – risk averse, bold – shy, or neophobic – neophilic) (Wilson & Godin, 2009). When behaviours are consistent within, but vary between individuals across a range of situations or contexts, they can be defined as 'personality traits' or 'behavioural syndromes' (Realé et al., 2007; Sih et al., 2004a). An example of a behavioural syndrome is seen in the funnel web spider, *Agelenopsis aperata,* where more aggressive individuals show an increased tendency to attack both prey and conspecifics, and emerge quicker from a shelter after a stimulated predator attack (Riechert & Hedrick, 1993). However, consistent individual differences in behavioural types or behavioural syndromes may not be consistent across different populations of the same species. For example, stickleback populations from streams have been found to be bolder than stickleback populations from ponds (Alvarez & Bell, 2007). Furthermore, in the three-spined stickleback, *Gasterosteus aculeatus,* one population showed a behavioural syndrome linking aggression, boldness and activity, whereas another population did not (Bell, 2005). As components of behavioural syndromes appear to be heritable (Dingemanse et al., 2002; Drent et al., 2003) and may also be programmed by early life experiences, such as hormonal, nutritional or maternal effects (Arnold et al., 2007; and see review by Sih et al., 2004b), individuals may become specialized in behaviours that then affect their fitness, such as foraging (Herborn et al., 2010), dispersal (Dingemanse et al., 2003), or traits associated with helping such as territory defence and maintenance (Bergmüller & Taborsky, 2007; Schürch & Heg, 2010). So, behavioural syndromes may reflect ecologically significant variation between individuals and populations that may then affect fitness related traits (Realé et al., 2007; Sih et al., 2004b) such as helping.

The aim of this project was to assess whether relatedness and/or consistent individual differences in behaviour influenced the amount of helping behaviour shown by helpers in breeding groups of *Neolamprologus pulcher* . *N. pulcher* is a cooperatively breeding African cichlid species endemic to Lake Tanganyika. This species lives in social groups governed by a strict dominance hierarchy, where a territory contains a dominant breeding pair and 1-14 helpers who vary in size, sex and relatedness (Balshine et al., 2001; Dierkes et al., 2005; Taborsky & Limberger, 1981). Helpers aid breeders by cleaning and fanning eggs, keeping

the breeding shelter free of sand and debris, and defending the territory and other group members against predators and intruding conspecifics (Taborsky, 1984; Taborsky & Limberger, 1981). Helping is costly, as helpers suffer reduced growth (Taborsky, 1984), lost mating opportunities (Taborsky & Limberger, 1981) and greater energy expenditure when helping, compared to normal swimming (Grantner & Taborsky, 1998). To counteract these costs, helpers can receive direct fitness benefits through group living; for example, increased survival due to having access to a shelter and protection from larger group members (Taborsky, 1984). In addition, male helpers may be able to parasitize the reproduction of the breeders (Dierkes et al., 1999; Heg et al., 2006). Female helpers occasionally may breed in addition to the dominant female (Heg & Hamilton, 2008), and often go on to inherit the breeding territory (Dierkes et al., 2005; Stiver et al., 2006). Additionally, related helpers are predicted to accrue indirect fitness benefits through kin selection (Brouwer et al., 2005; Taborsky, 1984). Previous work on *N. pulcher,* found that individuals can recognise kin via phenotype matching (see chapter 2 and Le Vin et al., 2010a), so helpers should be able to assess relatedness to breeders and could adjust their helping behaviour accordingly. However, previous studies on *N. pulcher* have found mixed results on the effects of relatedness on helping effort. For example, in the field, helpers related to the breeding female and unrelated to the breeding male were found to carry out the most defence. In the laboratory, in contrast, helpers unrelated to both breeders carried out the most helping in the form of territory defence and territory maintenance, compared to helpers related to both breeders (Stiver et al., 2005). However, this study only investigated natural helping levels, so there were no manipulations to standardise helping effort. Therefore, there is likely to have been substantial variation between groups, in the field and the laboratory, in the amount of helping required. Recent studies have also started to investigate the role of behavioural types and behavioural syndromes in determining helping performance in *N. pulcher* (Bergmüller & Taborsky, 2007; Schürch & Heg, 2010). However, these studies have found differences between populations in the behavioural syndromes exhibited. Further, they found differences in the effects that the behavioural traits had on helping effort. More importantly, in such a social species where relatedness between individuals differs within groups, these

studies did not assess the possible interactions between helper relatedness, their behavioural types and the amount of helping effort shown.

The aim of my experiment was to simultaneously assess the effects of relatedness and consistent individual differences in behaviour in *N. pulcher* on individual helping effort. I tested *N. pulcher* in a controlled laboratory set-up within standardized family groups that accounted for body size, familiarity and relatedness between individuals, but which were still representative of natural groups. I assessed two aspects of helping behaviour in *N. pulcher:* the amount of digging helpers carried out when the breeding shelter was filled in experimentally with sand (territory maintenance), and the amount of defence shown against a size-matched conspecific intruder (territory defence). Maintaining access to shelters by clearing away debris is an important helping behaviour as it provides a refuge in which to hide from predators (Taborsky, 1984) and aids survival of small offspring (Taborsky & Limberger, 1981). Defence of the territory from conspecifics is also vital to ensure that the territory is not taken over by other individuals (Taborsky, 1984). I addressed the following specific questions: 1) Is there variability between and consistency within individuals in their helping effort in either territory maintenance or territory defence? 2) Is there a correlation between the amount of territory defence and maintenance carried out; i.e., are some individuals generally more helpful than others? 3) Does relatedness to the dominant breeding pair affect the amount or type of helping shown? 4) Is there variability in individual aggressiveness, activity and risk-taking between individuals and are individual differences in these behaviours repeatable over time and hence behavioural types? 5) Are behavioural types correlated with each other in behavioural syndromes, or are they distinct axes of behaviour? 6) Do behavioural types predict the amount or type of helping effort performed by an individual?

3.3 Methods

3.3.1 Set up of social groups

Experiments were conducted at the University of Glasgow on the Lake Tanganyikan endemic cichlid species, *Neolamprologus pulcher*. Experiments

used adult *N. pulcher* that were supplied to us from the University of Bern, Switzerland. Some of these fish were wild caught and others, captive bred. The wild adults were caught at Nkumbula Island, near Mpulungu, Zambia in 2006. The captive bred adults were the offspring of wild fish caught at Kasakalawe, near Mpulungu, Zambia in 1996. These adults were known not to be inbred (see chapter 4). Prior to the experiment, all adults had bred at least once, producing F1 offspring that were also used in this experiment. These F1's were removed from their parents as eggs to prevent familiarity arising between family members. Further information on the breeding design used to create these F1's can be found in Le Vin et al (2010a) and chapter 2.

To investigate helping effort, social groups of *N. pulcher*, consisting of an adult male and female breeding pair, from my stock fish supplied by the University of Bern, and two juvenile F1 helpers were set up. One helper was the unfamiliar offspring of the breeders (related helper) and the other helper was unrelated and unfamiliar to the breeders (unrelated helper). Helpers were also unfamiliar with each other. Furthermore, these helpers had never helped before. Helpers were of a sexually mature size, > 35 mm standard length (SL) and were matched for SL (mean related = 39.12mm ± 0.46; mean unrelated = 38.94mm ± 0.49; paired *t*-test, $t = 0.41$, $N = 17$, $P = 0.69$), mass (mean related = 1.59g ± 0.07; mean unrelated = 1.55g ± 0.07; paired *t*-test, $t = 1.05$, $N = 17$, $P = 0.31$) and age (mean related = 305.94 days ± 14.98; mean unrelated = 317 days ± 13.56; paired *t*-test, $t = -1.45$, $N = 17$, $P = 0.17$). Thus, helpers should not have differed in their condition or experience and therefore, their ability to help.

Experimental tanks for the social groups measured 80 x 40 cm and 50 cm high, and were filled with approximately 140 litres of water. Tanks were oriented with the longest edge of the tank to the front, and the back of the tanks were covered with black plastic. This allowed the fish to be clearly viewed against a constant background, and gave the maximum view to an observer of the groups' behaviours. Water parameters were kept constant throughout the experimental period with temperatures of 26.8 ± 1°C, pH of 8-8.4 and a light regime of 13 hours light to 11 hours dark. Tanks were provisioned with a 1-1.5 cm layer of coral sand on the base, a foam filter and an airstone. Two breeding shelters made from terracotta plant pots cut in half provided shelters and breeding substrate, thus forming the territory. Shelters were checked daily for eggs,

which were counted and removed. The shelter was then replaced with a new shelter without eggs, as breeders have been observed to be more aggressive towards helpers when a new clutch is present (Taborsky, 1985). Also, I wished to control for differences in breeding between experimental groups. Two clear plastic tubes were also suspended at the top of the tanks, as refuges for fish receiving aggression from group members. Prior to, and throughout the experimental period, fish were fed *ad libitum* on a mixture of a commercial dry cichlid food, frozen bloodworm and frozen Daphnia, once daily.

Before being introduced to the experimental tank, both helpers and breeders were anaesthetised in a Benzocaine solution and SL and mass recorded. For identification, each fish was uniquely marked by fin clipping. To minimise breeder aggression, the two helpers were first introduced to the tank and allowed to acclimate for 24 hours (Dik Heg personal observations). The breeders were then introduced and the group were allowed to acclimate for a further 24 hours. During this time, all fish were watched closely to ensure that they were in good health and that aggression was not prevalent. Helpers were perceived to be accepted into the group when they had free access to swim around the tank, including in and around the breeding shelters. Fish that were not accepted hid in the refuge tubes and were chased by the breeders when they approached the breeding shelters. When helpers were not accepted into their group, I tried to encourage their acceptance by restraining the breeders for 17-24 hours in mesh cages within the experimental tank, so that the helpers had free access to all areas of the tank but with the breeders still present. Breeders were then removed from the cage and allowed free access to the tank, and the group was re-assessed to see if the helpers had been accepted. This method was used for 17 of 24 family groups I attempted to set up, and was successful on 12 occasions. In total, out of the 24 groups initially set up, 17 groups had helpers that were accepted by the breeders. Groups were together for a minimum period of 72 hours before any experiments were carried out. I carried out helping experiments and assessment of aggression levels of the helpers on all 17 groups, and on 14 of these groups I also carried out activity level and risk-taking assessments. Three groups did not have activity or risk-taking measures due to technical problems with the experimental set up. Measures of aggression shown by individuals were assessed within the social group, whereas measures of risk-

taking and activity were measured after the helping trials had been completed and the breeders had been removed from the tank. All behaviours were assessed by an observer blind to the relatedness of the helpers to the breeders.

3.3.2 Assessment of helping experiments

General protocol

Before helping experiments began, and on the days between helping trials, 10 minute behavioural observations of the fish were carried out. During this time I was able to check whether helpers were still accepted within the group.

Two helping experiments were conducted, during which the amount of territory maintenance and the amount of territory defence shown by individuals were quantified as measures of their helping effort. Each experiment consisted of three trials, from which a mean helping effort for territory maintenance and territory defence were calculated for each helper. In three cases, the full three maintenance and defence trials were not carried out due to helpers being rejected from the group, after the trials had taken place. In the first case, one territory defence and three maintenance trials were carried out, in the second case, the full three defence trials were carried out and one maintenance trial, and in the third case two defence and two maintenance trials were carried out. These data were still used in my analysis, as helpers were fully accepted within the group at the time of the trials. Territory maintenance and defence trials were carried out in a random order and there was at least 48 hours between subsequent trials. Before any observations, fish were allowed a 3 minute acclimation period from when the observer either entered the room and sat behind the screen or filled in a shelter with sand or introduced an intruder. My own personal observations as well as those of Bergmüller et al, (2005b) found that fish resume normal behaviour after this time period. This helped to minimise the effects of any disturbance caused to the fish by the observer or manipulations.

Territory maintenance trial

Before each trial, behavioural observations of the fish were carried out for 10 minutes as detailed previously, to ensure that helpers were accepted and to assess aggression levels prior to the trial. A standardized helping test of territory maintenance was carried out by manually filling in one of the two breeding shelters with sand to a set level (to the top of the breeding shelter) to assess individual digging effort. The group's digging behaviour was then recorded on a video camera for 50 minutes, and later scored for helping behaviour by an observer blind to the identity of the group members. On the video, the observer found the point when helpers began digging or carrying sand away from the shelter. For the next 10 minutes, the number of times each helper dug using either the body or mouth to move sand and carrying sand away from the shelter in the mouth was counted (Grantner & Taborsky, 1998). Territory maintenance was scored as the total number of digging and carrying acts per helper over the 10-minute observation period. Mean territory maintenance was then calculated across the three trials.

Territory defence trial

Again, before the territory defence trial started, a 10 minute behavioural observation was carried out to ensure that helpers were accepted into the group and to assess individual aggression levels. Territory defence trials consisted of an intruder phase and a control phase, which were presented in a random order. An unfamiliar, unrelated, size matched conspecific was introduced to a glass jar (1.2L) with a perforated lid to allow water exchange, for at least an hour before the trial to allow it to acclimate. During the intruder phase, the jar containing the intruder was then introduced into the centre of the experimental tank in between the two breeding shelters. Fish were then observed for 10 minutes. During the control phase, an empty jar was placed in the tank and the resident fish were observed for 10 minutes. The number of defensive behaviours shown towards the intruder in the jar or the empty jar was recorded: approaching the jar with opercular spread, biting the jar, swimming at the jar in a head down position and fast swimming at the jar, equivalent to ramming. Territory defence was scored as the number of defensive behaviours shown in the intruder phase minus the number of defensive behaviours shown in the control phase. This

controlled for levels of aggression shown towards an object (the jar) introduced into the territory, as opposed to a conspecific.

Assessment of behavioural types

Three behaviours were measured in *N. pulcher*: aggressiveness, activity level and risk-taking. Measures of helper aggressiveness were recorded during the 10-minute behavioural observations carried out before, and on the days in between, helping trials. These recorded the amount of aggressive behaviour shown by individual helpers towards other group members and from here on I call this 'within-group aggression'. In total, nine observation periods of within-group aggression were recorded: three measures in general observations where no trials took place afterwards, three measures before a territory maintenance trial and three measures before a territory defence trial. Aggressive acts recorded included: biting, chasing and approaches with opercular spread (Bergmüller & Taborsky, 2005). This gave me measures of within-group aggression for both the related and the unrelated helper when in a social group. For each of these different contexts a mean level of within-group aggression could be calculated for each individual.

After all helping trials had been carried out, the breeders were removed from the experimental tank. Helpers were then allowed 24 hours to acclimate without the breeders present. The experimental tank was divided into 5 vertical zones along the length of the tank (80 x 50 cm), each measuring 16 cm wide by 50 cm high and numbered one to five from left to right. Zones were marked out with 0.5 cm wide waterproof tape just after the breeders were removed. Two refuges at the top of the tank were located in zones one and five and two terracotta flower pot breeding shelters were located in zones two and four. Following this, on consecutive days each helper's activity levels in a familiar environment and risk-taking to a novel object were investigated, within the experimental tank. To obtain a measure of activity levels in a familiar environment for each fish, they were allowed to acclimate for 3 minutes following the observer moving behind the screen. Then the number of movements between the five zones was recorded for one helper for 10 minutes. Next, a 10-minute observation was carried out on the second helper. The order that helpers were observed was

randomised. Observations were carried out twice over two days for each helper so that a mean activity level could be calculated.

To assess risk-taking, helpers were exposed to two novel object trials; either a purple plastic popper (2.5cm height, 5cm diameter) or a red Buddha figurine (5cm height, 3.5cm width). Here, one novel object was placed in the centre of the tank between the two breeding shelters, in zone three. The latency to approach the novel object within two body lengths of the focal fish was recorded. Latency to approach the novel object was measured as the time from when the observer placed the novel object in the tank and sat immediately behind the screen. Fish had a maximum of 10 minutes to approach the object and if they did not approach it within this time they were scored with a latency of 600 seconds. The novel object was then removed and the following day a second risk-taking trial was carried out with the other novel object. The order of object presentation was randomised. Activity level observations were carried out before risk-taking trials as the disturbance caused by introducing a novel object may have affected activity levels.

3.3.3 Data analysis

All data were analysed using SPSS version 15.0. Data were checked for normality and homogeneity of variance, and where these assumptions were violated, non-parametric tests were carried out. Additionally, all tests were two-tailed. Firstly, I assessed that helping behaviours were repeatable, by carrying out single factor ANOVA's and calculating repeatability using the equation, r = S2A/(S2 + S2A) to generate the r value (See Lessells and Boag, 1987). Where the assumptions of sphericity were violated, I made corrections using the corresponding significance value (using the Greenhouse-Geisser, Huynh-Feldt or lower bound significance values). To test whether relatedness affected helping, a Wilcoxon matched pairs signed-ranks and a paired t-test were carried out, respectively, for territory maintenance and defence. I carried out analyses to ensure that the behaviours measured were repeatable, using the same method as above. I assessed if there were any differences between relatedness and the personality traits measured using Wilcoxon's signed-ranks tests. Further, I tested for correlations between personality traits using Pearson and Spearman's rank

correlations. Finally, I investigated the effect of personality traits on the amount of helping shown, by carrying out GLM's that also controlled for the relatedness of the helpers. I looked for both main effects and interactions, and removed non-significant interactions and then non-significant main effects from the GLM's in a backwards stepwise procedure. When investigating whether within-group aggression of helpers affected the amount of territory maintenance shown I used the amount of within-group aggression shown in the observations before the territory maintenance trial took place. Similarly, for territory defence I used the levels of within-group aggression shown by individuals in the observations before the territory defence trial.

3.4 Results

3.4.1 Variability and repeatability in helping efforts

I found that there was individual variability in the amount of helping carried out. For territory maintenance, the amount of helping ranged from 0 - 58.33 acts of digging and carrying per 10 mins (mean = 7.43 ± 2.07) and for territory defence the amount of defence shown ranged from 0 - 28.67 defensive acts per 10 mins against a conspecific intruder (mean = 7.88 ± 1.41). I also found that territory maintenance (ANOVA; $F_{29, 60}$ = 3.01, P < 0.001, r = 0.40) and territory defence (ANOVA; $F_{29, 60}$ = 5.31, P < 0.001, r = 0.59) were repeatable within individuals across trials. So, there is variability between individuals and consistency within individuals in their helping efforts, so they could be termed helping behavioural types.

3.4.2 Correlations between territory defence and digging behaviours

The amount of territory defence performed was correlated with the amount of territory maintenance an individual performed (Spearman's rho; r_s = 0.34, N = 34, P = 0.05; see Figure 3-1). Thus, some individuals were generally more helpful than others.

3.4.3 Effects of relatedness on helping

The helpers related to the breeders did not perform a greater amount of territory maintenance, in the form of digging and carrying sand away from the breeding shelter then the unrelated helper (Wilcoxon signed-ranks test; z = -0.26, N = 17, P = 0.82; see Figure 3-2a). Relatedness also had no effect on the amount of territory defence helpers carried out towards a conspecific intruder (Paired t-test; t = -1.32, N = 17, P = 0.21; see Figure 3-2b). Furthermore, when I calculated total helping effort (both territory maintenance and defence), the related and unrelated helpers did not differ in the total amount of help carried out (Wilcoxon signed-ranks test; z = -0.09, N = 17, P = 0.94). However, it is interesting to note that although there was no difference in the mean amount of helping carried out by related and unrelated helpers, there was a lot more variation in the amount of helping carried out for both territory maintenance and territory defence by the unrelated helpers compared to the related helpers.

3.4.4 Variability and repeatability in behavioural types

Relatedness to the breeding pair had no effect on an individual's behavioural type. Thus, related helpers were not more aggressive (Wilcoxon signed rank test; z = -0.23, N = 17, P = 0.83), active (Wilcoxon signed-ranks test; z = -0.22, N = 17, P = 0.86) or risk-prone (Wilcoxon signed-ranks test; z = -0.66, N = 17, P = 0.54) than unrelated helpers.

I found that there was variability between individuals in their behaviour (see Table 3-1), but that behaviours were repeatable within individuals. For levels of within-group aggression during general observations when no trial took place afterwards (ANOVA; $F_{27, 56}$ = 2.63, P = 0.001, r = 0.36), levels of within-group aggression before the maintenance trials (ANOVA; $F_{29, 60}$ = 2.70, P < 0.001, r = 0.36), levels of within-group aggression before the intruder trials (ANOVA; $F_{29, 60}$ = 4.44, P < 0.001, r = 0.53), activity levels (ANOVA; $F_{27, 28}$ = 8.36, P < 0.001, r = 0.78) and risk-taking (ANOVA; $F_{27, 28}$ = 2.19, P = 0.02, r = 0.37), individuals did not vary between trials. Therefore, I calculated a mean behavioural type per individual for later analysis. As individuals showed consistent individual

differences in their behaviours, I can conclude that my measures of within-group aggression, activity and risk-taking constitute behavioural types.

3.4.5 Correlations between behavioural types

I found no correlations between my behavioural types (see Table 3-2). Although there was a non-significant trend for the mean within-group aggression before an intruder trial to correlate with activity levels (Spearman's rho; r_s = 0.36, N = 28, P = 0.06), I did not find this trend consistently in any of the other correlations of within-group aggression and activity (see Table 3-2). Thus, within-group aggression, risk-taking and activity do not correlate, as would be expected if they constituted a behavioural syndrome.

3.4.6 Effects of behavioural types on helping

The amount of within-group aggression shown by a helper before a territory maintenance trial was not related to the amount of territory maintenance it carried out (GLM; $F_{1, 32}$ = 1.81, P = 0.19; see Figure 3-3a). There was also no effect of helper activity levels on the amount of territory maintenance carried out (GLM; $F_{1, 26}$ = 1.74, P = 0.20; see Figure 3-3b). However, there was a non-significant trend for helpers that took more risks, in approaching a novel object, to carry out more digging acts (GLM; $F_{1, 26}$ = 3.76, P = 0.06; see Figure 3-3c) than more risk-averse helpers.

Helpers' within-group aggression prior to a territory intruder trial was positively related to the amount of intruder defence they carried out (GLM; $F_{1, 32}$ = 27.16, P < 0.001; see Figure 3-4a). More active helpers carried out more defence against a conspecific intruder than less active helpers (GLM; $F_{1, 26}$ = 4.44, P = 0.04; see Figure 3-4b). Finally, risk-taking was also found to be positively correlated to the amount of intruder defence carried out (GLM; $F_{1, 25}$ = 13.12, P = 0.001; see Figure 3-4c). I also found that relatedness was significant in this model, as it was included as a factor (GLM; $F_{1, 25}$ = 4.58, P = 0.04). However, contrary to this, I had already found that relatedness did not affect the amount of territory defence carried out (Figure 3-2b), and that individual levels of risk-taking were not affected by whether the individual was related or unrelated to the breeders. There was extensive variation in the amount of defence carried out by the

unrelated helper compared to the related helper (Figure 3-2b). And, although the assumption of equality of variances was not violated in this model, this large difference in variances may have made the model unreliable and thus gave a false positive effect of relatedness. Thus, although within-group aggression, risk-taking and activity levels do not correlate together in a behavioural syndrome, they do all individually correlate with territory defence.

Table 3-1. Variation in behavioural types across individuals. Table shows the full range of behaviour expressed between individuals, over the three observations for aggression and the two observations of activity and risk-taking. The mean amount of each behaviour is also shown across all individuals ± S.E.

Trait	Range	Mean ± S.E.
[1]Aggression - general observation	0 – 5.50	1.28 ± 0.24
[1]Aggression - before maintenance	0 – 7.67	1.19 ± 0.26
[1]Aggression - before defence	0 – 8.00	1.11 ± 0.28
[2]Activity level	2 – 70.50	35.68 ± 3.20
[3]Risk-taking	15.50 – 600.00	281.89 ± 36.44

[1]Aggression = Mean no. of within group aggressive acts (in general observations with no helping trial afterwards, general observations before a territory maintenance trial and general observations before a territory defence trial)

[2]Activity level = Mean no. of moves between zones

[3] Risk- taking= Mean latency to approach a novel object (secs)

Table 3-2. Lack of correlations between behavioural types. Table shows details of correlations between the different behaviours investigated, the sample size for the correlation, the correlation coefficient and the significance value for the correlation. All correlations are Spearman's rank correlations.

Trait	N	Correlation coefficient	P
[1,2]Aggression in general observation Vs Activity level	28	0.26	0.19
[1,2]Aggression before maintenance Vs Activity level	28	0.28	0.15
[1,2]Aggression before defence Vs Activity level	28	0.36	0.06
[1,3]Aggression in general observation Vs Risk-taking	28	0.02	0.92
[1,3]Aggression before maintenance Vs Risk-taking	28	0.13	0.52
[1,3]Aggression before defence Vs Risk-taking	28	-0.09	0.66
[2,3]Activity level Vs Risk-taking	28	-0.15	0.46

[1]Aggression = Mean no. of within group aggressive acts (in general observations with no helping trial afterwards, general observations before a territory maintenance trial and general observations before a territory defence trial) in 10 minute observation period

[2]Activity level = Mean no. of moves between zones in 10 minute observation period

[3]Risk-taking = Mean latency to approach a novel object (secs) in 10 minute observation period

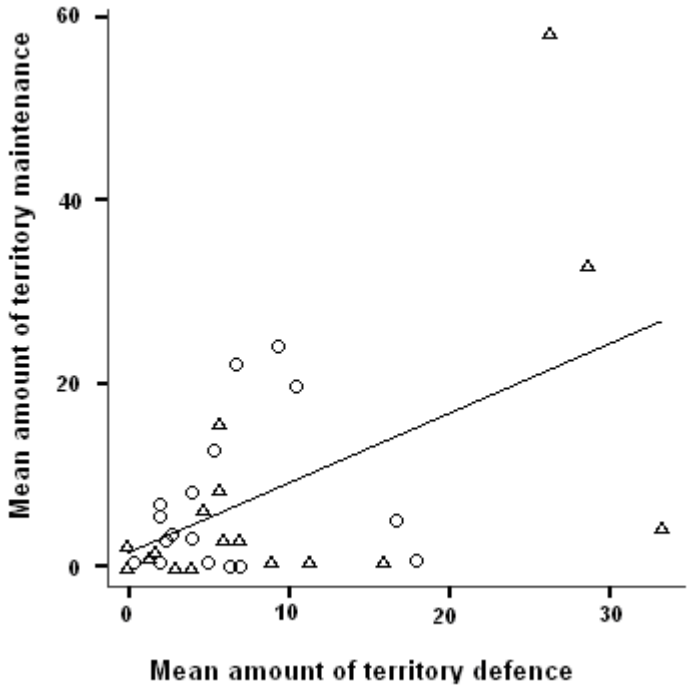

Figure 3-1. Positive correlation between territory maintenance (mean no. of digging events) and territory defence (mean no. of aggressive acts towards a conspecific intruder) during 10 minute observation periods, for related (open circles) and unrelated (open triangles) helpers combined. Individuals that carried out more territory maintenance also carried out more defence (P = 0.05).

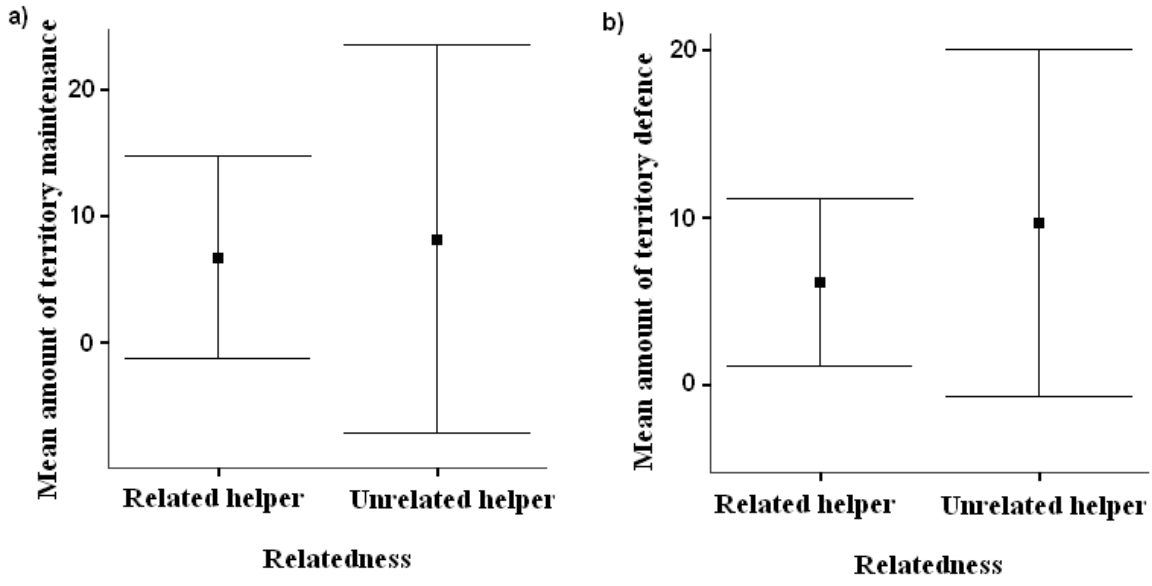

Figure 3-2. Comparison of helping effort between related and unrelated helpers. There were no significant differences in the mean amount of a) territory maintenance (mean no. of digging events) (P = 0.82) or b) territory defence (mean no. of aggressive acts towards a conspecific intruder) (P = 0.21) carried out by related and unrelated helpers during 10 minute observation periods. Error bars show the mean ± S.E.

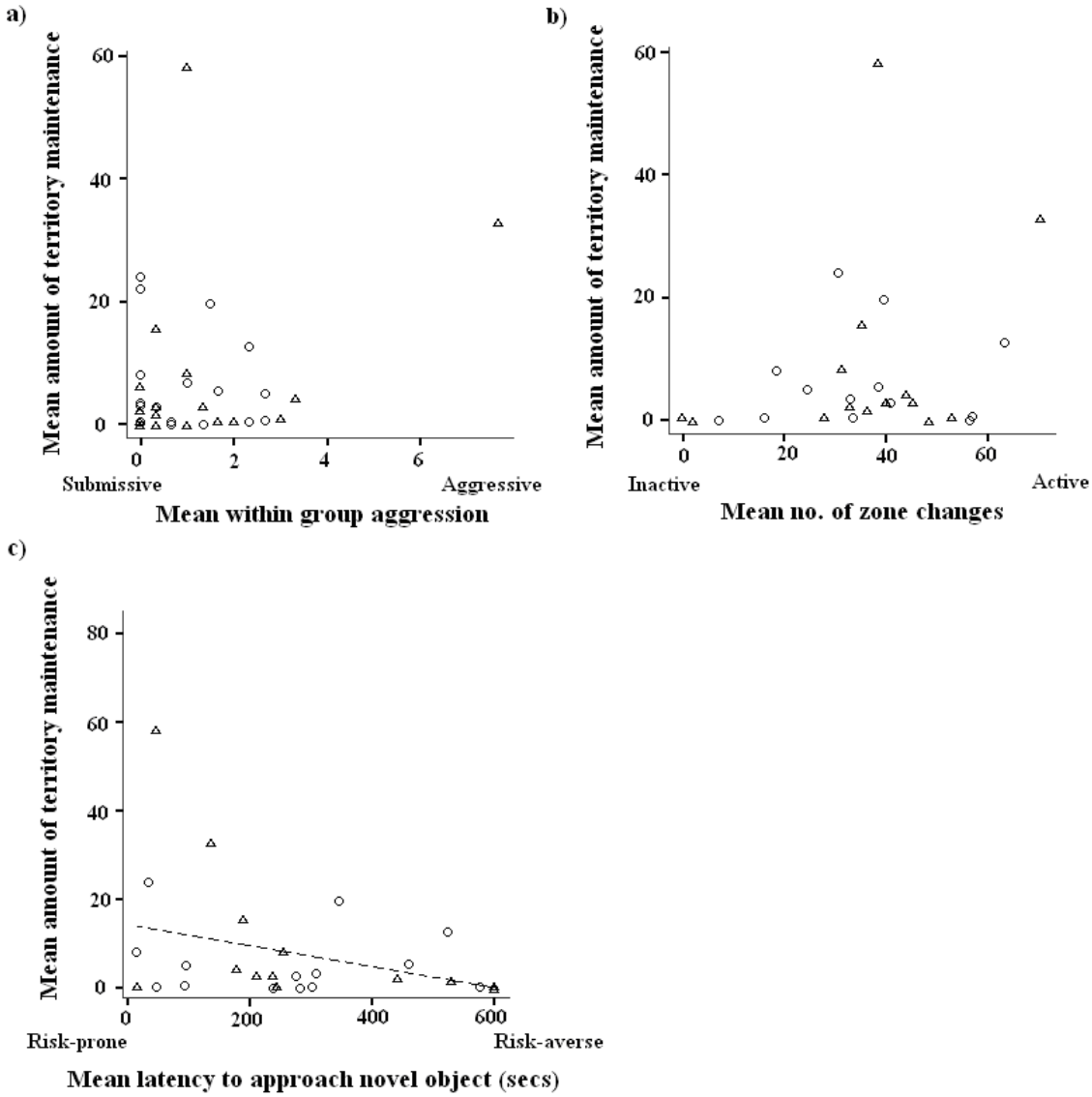

Figure 3-3. Relationships between behavioural types and territory maintenance helping effort. No significant relationships between helpers territory maintenance and a) within-group aggression (P =0.19); b) activity levels (P = 0.20); there was a non-significant trend between helpers territory maintenance and c) risk-taking (P = 0.06) in all cases scored as events per 10 minute observation. All figures show data for related (open circles) and unrelated (open triangles) helpers combined.

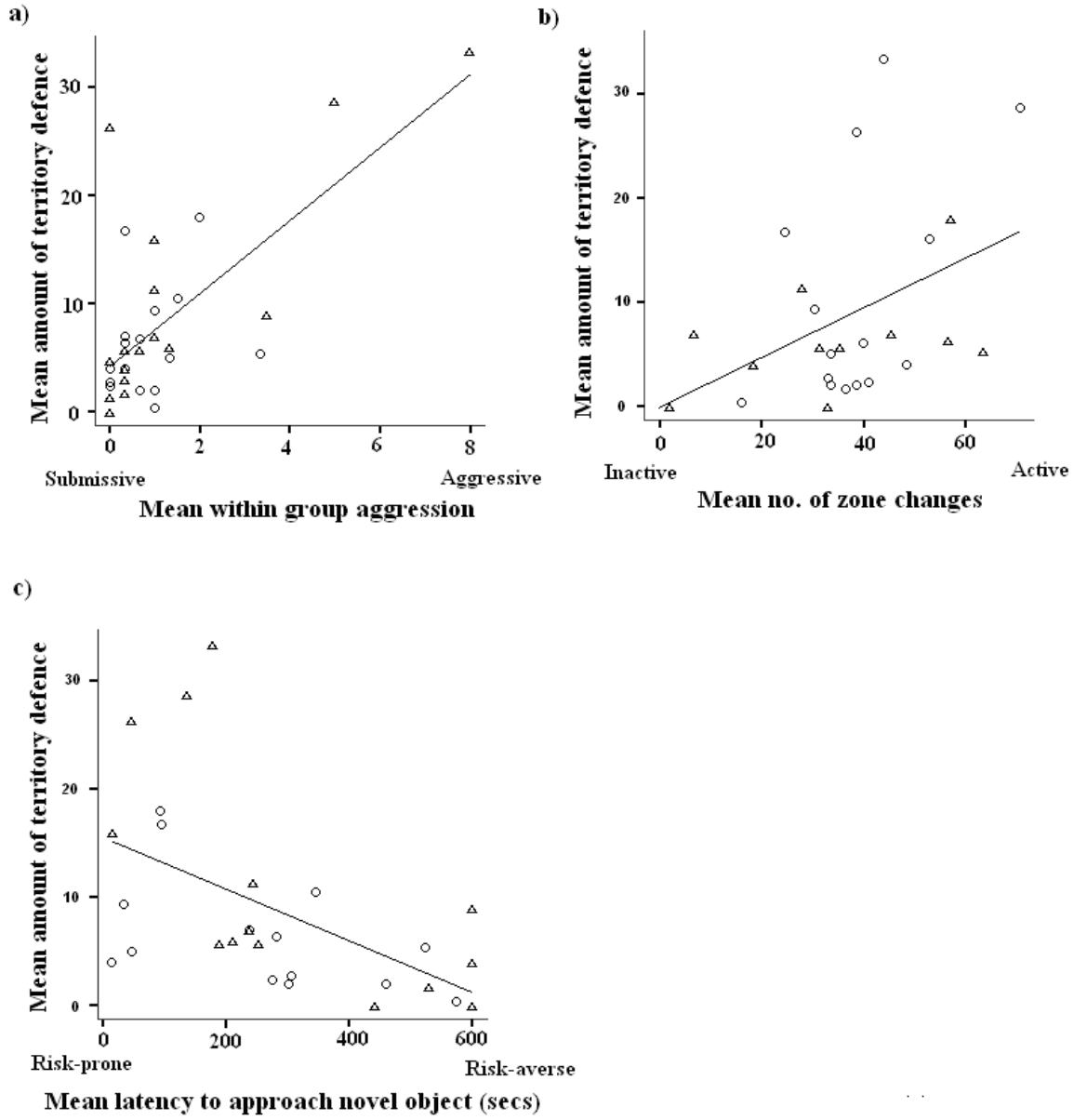

Figure 3-4. Relationships between behavioural types and territory defence helping effort. Significant relationships between helpers territory defence and a) within-group aggression (P < 0.001); b) activity levels (P = 0.04); and c) risk-taking (P = 0.001) in all cases scored as events per 10 minute observation. All figures show data for related (open circles) and unrelated (open triangles) helpers combined.

3.5 Discussion

Overall, I found that helping effort was variable between, but repeatable within individuals. Further, I found that some individuals were always helpful, with individuals that carried out more defence also carrying out more digging. Although I have previously demonstrated that *N. pulcher* can recognise kin (see chapter 2 and Le Vin et al., 2010a), relatedness to the breeding pair had no effect on the amount of helping, in terms of territory maintenance and defence performed. Further, I found no evidence that related helpers carried out more risky territory defence or that unrelated helpers did more, less risky, territory maintenance. Within-group aggression, activity and risk-taking were also found to be variable between, but repeatable within individuals, so can be classed as behavioural types. However, I did not find any correlations between these behavioural types. In contrast to relatedness, I found significant correlations between these behavioural types and helping effort, with more aggressive, risk-prone or more active individuals carrying out more territory defence than more submissive, risk-averse or inactive individuals. Furthermore, there was a non-significant trend for more risk-prone helpers to also carry out more territory maintenance than risk-averse helpers. So, it would appear that individual differences in behavioural types have more effect than relatedness, on the amount and type of helping an individual carries out.

I found no difference between related and unrelated helpers in their contribution to helping in both territory maintenance and defence in my laboratory set up. Previous work by Stiver et al (2005) on *N. pulcher* in the wild, looked at the effects of relatedness on helping under natural conditions. They found that helpers related to the breeding female, and helpers unrelated to the breeding male showed more territory defence. However, there was no such correlation between relatedness and overall helping effort (combining territory defence and territory maintenance). This seems to support my findings that both related and unrelated helpers help. However, Stiver et al. (2005) found that within cooperatively breeding groups in the wild, helpers were on average only related to the breeding female at the level of first cousins ($r = 0.125$) and unrelated to the breeding male. Therefore, their conclusion that the related helpers may be helping for kin-selected benefits, whilst unrelated helpers help

in order to 'pay to stay', may be overstated, as so few helpers (approx 16%) were first order relatives of the breeders within-groups. In an analogous laboratory study, Stiver et al (2005) found that unrelated helpers carried out more combined territory defence and maintenance than related helpers. This is contradictory to my work, as I found no effect of relatedness on combined helping effort or on separate measures of territory maintenance and defence. The inconsistencies between my results may be due to differences in experimental set up. In the laboratory, Stiver et al (2005) used groups that had been established for at least two years and contained varying numbers of either only related or only unrelated helpers, rather than a mixture of both, as is seen in natural groups. This may have had effects on group helping dynamics. In contrast, I standardised group size, familiarity and relatedness between group members, by having one related (r = approximately 0.5) and one unrelated (r = approximately 0) helper. Further, neither Stiver et al's (2005) field nor laboratory studies manipulated helping requirements for the fish. Instead they looked at natural levels of territory maintenance and defence against conspecific neighbours. Therefore, there is likely to have been substantial variation between groups in the quantity of helping required. By filling in shelters with sand or introducing a conspecific intruder, I provided a group with a standardised challenge to which the helpers could respond. As I found that related and unrelated helpers helped equally, it appears that kin selected benefits alone cannot explain the helping effort in terms of territory maintenance and defence in *N. pulcher*. However, it is possible that relatedness may influence other behaviours that were not studied in this experiment, such as fanning of eggs or defence of fry. Whilst related helpers receive both kin selected and direct fitness benefits, unrelated helpers receive only direct benefits. Thus, these direct benefits must be equal to the combined benefits the related helpers receive, or alternatively, unrelated helpers may have to 'pay to stay' more than a related helper to be tolerated by the breeders, as has been proposed by Kokko et al (2002). Evidence of helpers 'paying to stay' has been found in *N. pulcher* (Taborsky, 1985), although the studies have not taken into account relatedness. Helpers that were temporarily removed from the territory, and thus prevented from helping, increased their helping effort when they were returned to their group (Balshine-Earn et al., 1998), and helpers given access to other breeding territories, and hence given the opportunity to breed

independently, reduced their helping effort in their group (Bergmüller et al., 2005b). However, it is also possible that the kin selected benefits that the related helpers receive are negligible, and in fact the direct benefits of group living are driving helping effort regardless of relatedness (Clutton-Brock, 2002; Griffin & West, 2002).

Further, I found that relatedness had no effect on the type of helping carried out. Related helpers were not found to carry out more risky helping, such as defence against an intruder which carries the risk of injury (Balshine et al., 2001). Equally, unrelated helpers did not carry out a greater proportion of, less risky helping, such as territory maintenance. Individuals were also found to be consistent in their helpfulness, with individuals that carried out more territory defence also carrying out more territory maintenance. So, certain individuals may be pre-disposed to be helpful, perhaps because they more experienced in helping, are in better condition and/or are of a different behavioural type. Overall, it appears that it is individual differences in helper behaviour, rather than their relatedness to the recipient breeders that is driving helping behaviour in this species.

Aggression, activity and risk-taking were uncorrelated with each other. However, these three behavioural types were individually correlated with the amount of defensive helping effort shown, and could each be termed a behavioural syndrome (Sih et al., 2004a). However, I feel that this term is not appropriate here, as I would have expected to have also seen a correlation between the three behavioural types. Instead, consistent differences in an individual's behaviour seem to influence an individual's propensity to help in the form of territory defence, as further described below. I found that individuals that performed more aggressive acts towards their own group members were more likely to show aggression towards an intruder. Aggressive individuals within a social group may appear detrimental to the group, by potentially increasing competition for food, reducing growth and increasing the risk of injury to conspecifics (Huntingford et al., 2006). However, based on my data, there may be a trade-off, as these aggressive individuals should be tolerated because they could also be good at protecting the group from intruders. Accordingly, relationships between aggression and defence against a predator or conspecific intruder have been found in three-spined sticklebacks, *Gasterosteus aculeatus*,

and the funnel web spider, *A. aperta*, (Huntingford, 1976; Riechert & Hedrick, 1993). In *N. pulcher,* I found that individuals that took more risks carried out more defence against an intruder than risk-averse individuals. As risk-taking was a score of how quickly an individual approached a novel object, risk-prone individuals may be more inclined to approach an intruder, and hence, have more opportunities to help, than a risk-averse individual. Further, in other species risk-taking has been found to correlate positively with defence against predators, such as the pumpkinseed sunfish, *Lepomis gibbosus,* and bluegill sunfish, *Lepomis macrochirus* (Coleman & Wilson, 1998; Wilson & Godin, 2009). Therefore, it is possible that risk-prone *N. pulcher* may also show more defence against predators than risk-averse individuals. In addition, I found that more active individuals carried out more defence than inactive ones. It is possible that individuals that are swimming about their territory more may also be more likely to come across an intruder than a less active individual; consequently, they should carry out more defence. Carrying out defence against conspecific intruders is important in *N. pulcher,* as intruders may displace helpers and/or breeders (Taborsky, 1984). Therefore, having individuals within a group that are aggressive, more willing to take risks or be active, in addition to carrying out defence may be essential to maintain group stability.

In this study, I found no relationship between activity levels or within-group aggression and digging effort. Instead, I found a trend for individuals that took more risks to carry out more territory maintenance than risk-averse individuals. By manually filling in the shelter with sand, I caused a disturbance. It is possible that risk-prone individuals may also be more likely to investigate a disturbance on the territory than a risk-averse individual, and therefore come across the chance to help. Previous work by Schürch and Heg (2008) found a positive relationship between exploration and digging in females, and that exploration was positively correlated with risk-taking (which they termed boldness). In my study, however, I did not measure individual levels of exploration so I cannot directly compare.

I found that more aggressive, active or risk-prone individuals carried out more defence than risk-averse individuals. Although I did not find a correlation between aggression and risk-taking, it has been found to correlate in several species (Huntingford, 1976; Riechert & Hedrick, 1993), including *N. pulcher*

(Schürch & Heg, 2010). Schürch and Heg (2008) found a behavioural syndrome correlating aggression, risk-taking and exploration in both males and females after sexual maturity. However, I found no such behavioural syndrome in my population, even though the fish I used were also sexually mature. Further, Schürch and Heg (2008) found that more explorative females carried out more territory maintenance, whilst an earlier study found that more explorative males and females carried out more territory defence (Bergmüller & Taborsky, 2007). Consequently, this provides further evidence that behavioural syndromes, and individual behavioural types, can differ between populations of the same species, as has been described in sticklebacks (Alvarez & Bell, 2007; Bell, 2005). Early life effects that an individual experiences may influence behavioural type (see review by Sih et al 2004b) and behavioural syndromes have been found to have a heritable component (Dingemanse et al., 2002; Drent et al., 2003). In *N. pulcher,* I have found that an individuals behavioural type can predict helping effort, and that some individuals are consistently more helpful than others, thus the helping behaviours I investigated could be classed as behavioural types, which has seldom been tested in cooperative species (but see English et al., 2010; Schürch & Heg, 2010). If individuals are consistent in their helping it is possible that may be predisposed to show certain types, or amounts, of helping behaviour, which could lead to stable levels of cooperation in groups (McNamara et al., 2004). However, in different populations the type or the amount of helping shown may be adaptive to that particular environment, which may explain why differences are seen between populations in the individual behavioural types and behavioural syndromes exhibited (Alvarez & Bell, 2007; Bell, 2005; Bergmüller & Taborsky, 2007; Schürch & Heg, 2010). To conclude, my study has shown that in *N. pulcher,* differences in behavioural types rather than relatedness to the breeding pair, had effects on the amount and type of helping effort shown.

Helping has been found to be costly in many species, (reviewed in Heinsohn & Legge, 1999) including *N. pulcher* (Taborsky, 1984; Taborsky & Grantner, 1998). Individuals that differ in body condition may differ in their ability and/or willingness to help. However, as all of the helpers used in my experiment were fed on a standardized diet from birth, and each pair of related and unrelated helpers was matched for SL and mass, they should not have differed significantly

in condition, or their ability to help. The sex of my helpers was unknown, which could have had influences on results. Female helpers have been found to enter the breeding shelter more often, and show more brood care than male helpers (Heg et al., 2008b; Stiver et al., 2005). However, as previously discussed, Stiver et al's (2005) study did not test helping effort under standardised conditions, or control for sex within groups. Therefore, this result may be an artefact of the sex ratio of the groups studied. In contrast, Mitchell et al (2009), found that male subordinates impose a higher cost on breeder males than female subordinates, as dominant males have to show more aggression towards subordinate males who may parasitize reproduction. Therefore, male helpers may be expected to help more to counteract their costs, particularly if individuals have to 'pay to stay'. Another study, in contrast, found that helper sex had no effect on either territory defence or maintenance (Bruintjes & Taborsky, 2008). Consequently, although the sex of my helpers was unknown, it seems unlikely that this would have consistently affected my results.

In conclusion, I found that individual differences in behavioural types rather than relatedness to the dominant breeders within a social group were a better predictor of helping effort in *N. pulcher*. Overall, aggressive, risk-prone or active individuals showed more defence against intruders than submissive, risk-averse and inactive helpers, whilst more risk-prone helpers showed a trend to dig more than risk-averse helpers. Further, my study adds to the growing evidence that individuals show consistent behavioural types, and that these and their associated behavioural syndromes can differ between populations of the same species. My study expands upon previous work investigating the effects of relatedness and consistent individual differences in behaviour on helping in *N. pulcher,* and highlights the importance of considering multiple factors when investigating complex behaviours, such as helping, in socially living species.

Chapter 4: Adult kin recognition and inbreeding in the cooperatively breeding cichlid, *Neolamprologus pulcher.*

4.1 Abstract

Being able to recognise kin can bring fitness advantages to individuals, particularly in socially living species in which groups can contain related as well as unrelated individuals. These advantages may change with age; for example, juvenile individuals may choose to associate and cooperate with kin, whereas when sexually mature, they may avoid kin to prevent inbreeding. Here, I investigated the kin recognition capabilities in adults of the cooperatively breeding African cichlid, *Neolamprologus pulcher*. Further, I investigated whether *N. pulcher* actively avoided inbreeding and the consequences of pairing with siblings over non-siblings. Microsatellite analysis confirmed that the original stock fish that sired the sibling and non-sibling groups for this study were not inbred, which could have confounded results. In standard two-way choice trials, I found that female *N. pulcher* preferred to associate with unfamiliar brothers over male non-kin. Male *N. pulcher* showed no overall preference for associating with sisters or female non-siblings. Maximum likelihood relatedness scores between focal and stimulus fish did not correlate with strength of preferences for kin or non-kin. The size of facial stripes, a phenotypic trait that varies between individuals of *N. pulcher*, was also assessed to establish if they played any role in either kin recognition and/or as 'badge of status' in mate choice. In both males and females, the size of the stimulus fish's facial stripes did not influence the preference of the focal fish. Individuals were then paired in a cross-over breeding design, with either an opposite sex sibling or non-sibling in a randomised order. Relatedness scores between individuals had no influence on the propensity of individuals to breed. However, both male and female *N. pulcher* showed a trend for sibling pairs to have higher hatching success rates than non-sibling pairs. Therefore, in *N. pulcher*, inbreeding does not appear to be detrimental. Since individuals were just as willing to mate with non-relatives as relatives, breeding might be predominantly opportunistic rather than strategic. In the wild, other factors, such as sex-biased dispersal and regular

breeder replacement on territories, may further reduce the occurrences of close inbreeding in this species.

4.2 Introduction

Kin recognition is an internal, unobservable process that allows animals to classify conspecifics as being either kin or non-kin, whilst kin discrimination is the differential treatment of conspecifics dependent upon the degree of genetic relatedness between them (Holmes & Sherman, 1983; Waldman, 1988). Kin recognition is widely thought to be a learned process (Hepper & Cleland, 1998), with individuals either becoming familiar with the kin they are brought up with, or by some form of phenotype matching whereby they create a recognition template against which to compare others (Holmes & Sherman, 1983; Lacy & Sherman, 1983). Thus, studies of kin recognition need to control for familiarity, as well as relatedness between test subjects. Kin recognition has been found in a range of taxa (Arnold, 2000; Mateo & Johnston, 2000; Neff & Sherman, 2005; Whitehorn et al., 2009). However, few studies have explored how individuals recognise their kin. Choices to associate with kin may also change with an individual's age. For example, juveniles may prefer to cooperate and spend time with relatives to gain kin-selected benefits, whilst adults may wish to avoid kin in order to prevent inbreeding (Waldman, 1988).

Inbreeding is potentially problematic for individuals as it can lead to an increase in homozygosity and the expression of deleterious recessive alleles, which can reduce fitness traits such as fertility, offspring survival, body size and competitive ability (reviewed in Keller & Waller, 2002). Accordingly, a number of studies have found evidence that inbreeding can be avoided by being able to recognise kin (Archie et al., 2007; Gerlach & Lysiak, 2006). The chance of inbreeding may also be reduced indirectly by sex-specific dispersal, delaying maturation, or extra-pair copulations with unrelated individuals when paired with kin (Pusey, 1987; Pusey & Wolf, 1996). Inbreeding can pose a particular problem in group-living species, especially where dispersal is limited. This is particularly true for cooperatively breeding species, in which groups often include related individuals (Dierkes et al., 2005; Russell & Hatchwell, 2001; Stacey & Koenig, 1990). Recent work by Jamieson et al. (2009) found that

cooperatively breeding bird species generally avoid breeding with relatives, whilst birds breeding in single pairs breed randomly in accordance with relatedness to their breeding partner. Therefore, mechanisms of kin recognition may be more important and consequently, more finely tuned in these socially rather than solitary living species. Previously, before advancements in molecular techniques, trying to assess relatedness and the amount of inbreeding within populations was problematic and relied on pedigree data. Collecting pedigree data, particularly from wild populations, not only takes a long time to collect, but requires certainty of paternity if they are to be reliably interpreted (Keller, 1998). The use of molecular techniques, such as microsatellite analysis, have made assessments of relatedness between individuals much faster and simpler (Queller et al., 1993). Any two siblings should share, on average, 50% of their genes with each other. However, independent assortment during meiosis means that some individuals will have more than 50%, and some less, of their alleles in common with their siblings. The degree of allele sharing can give a relatedness score between individuals, allowing investigation into whether the degree of genetic relatedness between individuals influences or enhances either kin recognition and/or inbreeding.

Inbreeding may not always be detrimental to fitness, as outbreeding with individuals that are genetically very different can also be disadvantageous (Peer & Taborsky, 2005; Sagvik et al., 2005). So, inbreeding can help to preserve locally adapted genes (for review see Edmands, 2002). Breeding with relatives can even increase an individual's inclusive fitness if alternative mating opportunities are not lost by mating with a relative, and/or if the benefits of breeding with kin are greater than any costs associated with inbreeding depression (Kokko & Ots, 2006). High levels of inbreeding have been found in natural populations of dwarf mongoose, *Helogale parvula,* and naked mole-rats, *Heterocephalus glaber* (Reeve et al., 1990; Keane et al., 1996). In the greater white-toothed shrew, *Crocidura russula,* inbreeding does not affect fecundity or the future reproductive success of offspring (Duarte et al., 2009). Furthermore, active inbreeding has even been found to be advantageous in one species of cichlid, *Pelvicachromis taeniatus,* where sibling pairs were more cooperative and showed more parental care than unrelated pairs (Thunken et al., 2007). However, individuals may not actively choose to inbreed, but instead may hedge

their bets in order to optimise their long-term fitness (Philippi & Seger, 1989; Slatkin, 1974). For example, if breeding opportunities are limited, individuals may need to trade-off not breeding at all with inbreeding. Hence, inbreeding may be advantageous in some situations, or at least better than not breeding at all.

As mentioned, kin recognition abilities in terms of mate selection should be particularly important for group-living organisms such as cooperative breeders, like the cichlid *Neolamprologus pulcher*. This fish is endemic to Lake Tanganyika in East Africa and lives in social groups consisting of a dominant breeding pair and up to 14 related and unrelated helpers (Balshine et al., 2001). Helpers clean and fan eggs in the breeding shelter and help to defend the territory and other group members against predators and intruding conspecifics (Taborsky, 1984; Taborsky & Limberger, 1981). Previous work has found that juvenile *N. pulcher* recognised and preferred to associate with kin over non kin using phenotype matching (see chapter 2 and Le Vin et al., 2010a). Further, chemical rather than visual cues appeared to be most important in kin recognition in these sexually immature fish. In addition, recognition of familiar individuals, by sexually mature *N. pulcher,* using only visual cues, has been documented (Balshine-Earn & Lotem, 1998; Frostman & Sherman, 2004). Adult *N. pulcher* have two facial stripes on their operculum, which may aid in visual recognition of individuals (Duftner et al., 2007; Seehausen et al., 1999) and/or may function as a 'badge of status' involved in mate choice (Ferns & Hinsley, 2004; Setchell & Wickings, 2005). The juvenile *N. pulcher* used in the kin recognition experiments in chapter 2 had not yet developed their facial stripes (personal observations have found that facial stripes do not develop prior to four months after hatching). Therefore, among juveniles, facial stripes would not have influenced the decision to associate with either kin or non-kin whereas later in life they may be more influential in recognition of kin and/or mate choice.

Species that exhibit sex-biased dispersal should reduce the potential for inbreeding to occur, even without actual kin recognition. In the wild, *N. pulcher* generally exhibit male-biased dispersal (Stiver et al., 2004; 2007) whilst females often inherit their natal breeding territory (Dierkes et al., 2005; Stiver et al., 2006). Field studies have found that *N. pulcher* breed randomly with respect to relatedness, so neither actively inbreed nor avoid it (Stiver et al., 2008).

However, it should be noted that in Stiver at al.'s (2008) study, they could only examine allele sharing between pairs, and thus, they could not be certain that pairs with higher relatedness scores were actually genetically similar by descent (i.e. true relatives). So, although *N. pulcher* can recognise kin as juveniles (see chapter 2 and Le Vin et al., 2010a), it may not prevent them inbreeding as adults. This may be due to a lack of breeding opportunities (Kokko & Ots, 2006; Stiver et al., 2008), because breeding with relatives brings some advantages (Kokko & Ots, 2006), or perhaps because as adults, they are unable to assess kin. Whether or not *N. pulcher* can recognise kin and avoid them as adults or whether there are any fitness advantages to breeding with relatives has yet to be tested under controlled conditions.

4.2.1 Aims

The main aim of this study was to assess adult kin recognition and the effects of inbreeding in *Neolamprologus pulcher*. Thus, groups of unfamiliar siblings and non-siblings were created by breeding individuals from an adult stock of *N. pulcher*. The genetic origin of these adult stock individuals was unknown. Microsatellite analysis allowed relatedness scores, based on allele sharing between individuals, to be calculated. These were used to ascertain that the breeders were not closely related to each other, thus inflating the relatedness coefficients among the offspring and potentially confounding the result of preference tests. Using adult F1's, kin recognition experiments and breeding trials were then carried out, in which individuals were randomly assigned an opposite sex sibling or non-sibling as a potential mating partner. The following questions were addressed: 1) Do sexually mature *N. pulcher* show preferences for associating with kin or non-kin of the opposite sex? Further, where preferences are shown, do chemical and/or visual cues play a more important role in their preferences? 2) Do relatedness scores between the experimental and stimulus fish correlate with the strength of their preference? 3) Do the facial stripe size of stimulus fish influence mate preference? 4) Do relatedness scores between breeding pairs affect willingness to breed? 5) Is breeding success lower in sib-sib pairs compared to non-sib pairs?

4.3 Methods

4.3.1 General animal husbandry

The breeding stock of *N. pulcher,* used to create the sibling and non-sibling groups to assess kin recognition and the effects of inbreeding, came from two populations in two different areas of Lake Tanganyika in Zambia. One population of adults were wild-caught fish, from Nkumbula Island, near Mpulungu, caught in 2006 (from here on the 'wild caught' population). The other population were captive-bred fish, bred from wild-caught fish from Kasakalawe Bay, near Mpulungu, caught in 1996 (from here on the 'captive-bred' population). As these populations may have differed genetically from one another, they were paired separately within populations to breed to create F1 offspring.

Prior to breeding and experiments, all fish were kept in mixed-sex tanks under the following parameters, unless otherwise stated. The breeding stock adults were maintained in tanks ranging in size from 50 to 250 litres and stocking densities ranged from 3 to 27 individuals. The offspring from the crosses between the stock fish were kept in 50 litre tanks with their siblings, in groups ranging from 3-19 individuals. Tanks were provisioned with 1 - 1.5 cm of coral sand on the base, an airstone and a foam filter. The water temperature was kept in the range of 26.8 ± 1 °C, pH in the range 8 - 8.4 and a light regime of 13:11 h light:dark. All fish were fed once daily with either commercial dry cichlid food, frozen bloodworm or *Daphnia*.

4.3.2 Creating sibling and non-sibling groups

Prior to breeding, fish were anaesthetised using a benzocaine solution and sexed by examining the genital papilla. In males the genital pore is around the same size as the anus and in older or larger males there may be a small protrusion at the genital pore. In females, the genital pore is much larger than the anus and may be either round and slightly depressed or slit from left to right (Personal Communications Dr Dik Heg). Further, when sexing all fish also had their mass and standard length (SL) recorded. All fish had SL greater than 35 mm SL, as this is the size when *N. pulcher* are known to be sexually mature (Dierkes et al.,

1999). A single male and female from the same population were randomly paired and placed into a 140 litre tank (80 x 40 x 50 cm high) to breed to create F1 offspring. Breeding shelters were provided and consisted of two pieces of plastic pipe cut in half and two terracotta flowerpot halves. Each of the shelters had the inside surface covered with a thin flexible plastic layer that was clipped in place. Females laid eggs on the sides of the shelters, and so the plastic layer with the eggs attached could be removed easily. Shelters were checked for eggs every morning and evening. When eggs were found they were left with the breeding pair for a further 24 h, after which they were removed and the clutch counted. To count eggs the shelter was temporarily removed but kept submerged in water by placing it into a small 8 litre tank containing enough water to cover the eggs, to prevent desiccation. *N. pulcher* eggs range in colour from blue/green to a slight pink colour. Occasionally eggs are white or may have fungus growing on them. Personal observations have shown that these eggs do not hatch and are either diseased or infertile. In cases where white eggs were found, they were counted and then carefully removed from the clutch of eggs to prevent disease spreading. To create groups of familiar and unfamiliar siblings, each clutch was split into two. Each half clutch was transferred to a breeding net suspended in two different 50 litre tanks (48 x 27 x 35 cm high), with standard water parameters. An airstone was also placed in the breeding net to keep the eggs aerated. When the fry started to feed independently, at around 10 days old, they were fed on a combination of crushed flaked food and frozen *Cyclops* or *Daphnia* twice a day for the first three months and then they were fed once daily, as previously described. The number of fry surviving from a clutch was counted to assess the breeding success of these original breeders (survival assessed at approx 74 days old, Mean = 73.79 ± 1.52). These F1s were then used in the adult kin recognition and inbreeding experiments.

Fish were allowed three weeks to breed with a partner, as prior breeding trials had shown that if fish did not breed within this time they were unlikely to breed at all (Le Vin, personal observations). Individuals were then rested from breeding for a period of two weeks by removing their breeding shelters. After this period they were re-sexed and paired with another partner to breed (as above). Out of 56 breeding pairs established, 29 pairs produced a clutch of eggs. After breeding had finished, the adult breeding stock were anaesthetised and fin

clips taken. They were then genotyped at eight microsatellite loci, and relatedness scores between pairs were calculated, to assess whether there was any evidence of the population being inbred. If the population had been inbred, it could have exaggerated the relatedness coefficients among their offspring, which could have confounded the results of the kin recognition and inbreeding experiments.

4.3.3 Kin recognition of opposite sex adults

Overview of experiment

Using F1's, experiments were carried out testing adult male *N. pulcher* for preferences for associating with unfamiliar female kin over unfamiliar female non-kin, and testing female *N. pulcher* for preferences for associating with unfamiliar male kin over unfamiliar male non-kin. The experiment consisted of two tests: a matched-cues test and a mismatched-cues test, each of which was repeated to control for side bias. In total, four trials (two matched cues and two mismatched cues) were run over four consecutive days, in a randomized order, and using the same focal fish in each of the four trials. In the matched-cues test, the visual cues from the stimulus fish matched their chemical cues (chemical cues from A to X and B to Y; Figure 4-1). In the second trial of the matched-cues test, the sides on which the cues were presented were swapped to control for side biases. In the mismatched-cues test, the visual cues from the stimulus fish did not match their chemical cues. So, in one side of the tank the focal fish could view one stimulus group but simultaneously received the chemical cues of the other stimulus group and vice versa on the opposite side of the tank (chemical cues from A to Y and B to X; Figure 4-1). Again, I controlled for side bias by swapping the sides on which the stimulus fish were presented. From the mismatched-cues test the importance of chemical and/or visual cues in the preference for kin or non-kin of sexually mature *N. pulcher* could be assessed.

Before the experiment, all fish were sexed and swabbed to obtain DNA for microsatellite analysis. This was used to calculate relatedness scores, to investigate whether these correlated with the focal fish's preference for either the kin or non-kin stimulus fish. Experimental fish and stimulus fish were matched by standard length SL (Female experiments: range = 40-66 mm; mean =

52.64 ± 1.09; one-way ANOVA; $F_{2, 38} = 0.30$, $P = 0.74$. Male experiments: range = 36-65mm; mean = 49.21 ± 1.26 mm; one-way ANOVA; $F_{2, 32} = 0.62$, $P = 0.55$) and mass (Female experiments: range = 1.40 – 6.73 g; mean = 3.94 ± 0.25 g; one-way ANOVA; $F_{2, 38} = 0.27$, $P = 0.73$. Male experiments: range = 0.85 – 6.73 g; mean = 3.10 ± 0.26 g; one-way ANOVA; $F_{2, 32} = 0.31$, $P = 0.73$).

Analysis of facial stripes

Whilst anaesthetised, fish were also photographed for facial stripe analysis. Pictures were taken with a Panasonic Lumix DMC-FZ20 camera in TIFF format. Photographs were taken of the left lateral side of each fish against a background of 1 mm square graph paper. Facial stripes were then analysed using the programme ImageJ, zooming in on each image by 4:1 to ensure greater accuracy in measurements. By using ImageJ's measuring function, size could be calibrated by measuring along the length of one of the sides of a 1mm square on the graph paper and inputting the size as 1 mm to ImageJ. The area of each facial stripe was measured by drawing around the facial stripe using the polygonal selection tool and ImageJ then calculated an area (see Figure 4-2). This was repeated five times and a mean taken to increase accuracy. The maximum length (from top to bottom of the stripe) and breadth (measured at the widest part of the stripe) of each facial stripe was also calculated using the straight line tool. Again, five measurements and a mean were calculated (see Figure 4-2). Mean stripe area was used in all further analysis, as all measurements were found to be highly correlated (see Table 4-1). Stripe analysis was carried out by an observer blind to the behavioural results.

Experimental set up

Fish were then allocated to individual tanks (35 x 35 x 30 cm filled with 8 litres of water for the experimental fish and 19 x 18 x 22 cm filled with 3 litres of water for the stimulus fish) and allowed to settle for at least 22 h. The focal fish experimental tank was arranged adjacent to the two stimulus tanks containing an unfamiliar kin and unfamiliar non-kin stimulus fish of the opposite sex (see Figure 4-1). After this 22 h period, full water changes were done in each tank to remove any chemical cues that may have been produced as a stress response to being anaesthetised or any chemical residues of the anaesthetic itself. To

provide the stimulus water with chemical cues, a further 22 h acclimation period with the stimulus fish present in their tanks followed. Filters were not included in either the experimental or stimulus tanks to prevent removal of chemical cues. Therefore, the water quality was checked regularly to confirm that it remained within safe levels for the fish. All tanks were covered with card on three sides to prevent the fish being disturbed by our observations, and to prevent the stimulus fish from seeing each other. Removable card barriers were also positioned between the experimental and stimulus tanks to prevent the focal fish from seeing the stimulus groups during the acclimation period. The experimental tank was marked out with two 'preference zones' located adjacent to the front of each stimulus tank, each measuring 14 cm long by 12 cm wide. Between each of these preference zones was a 7 cm 'no preference zone', as was the rest of the tank (see Figure 4-1). Water was drawn from each stimulus tank, through peristaltic pumps, and dripped into the middle of each preference zone through silicone tubing, which we secured above the tank. Flow rate was set to 1.4 ml/min, which is sufficient to induce a reaction to a chemical stimulus (see chapter 2 and Le Vin et al., 2010a). Flow rate was checked regularly to ensure accuracy. Pilot studies with colour-dyed water showed that the water from each pump remained concentrated and mainly localized within the target preference zone. To observe the fish during the trials from a distance without disturbing them, a mirror was placed above the tank.

Kin recognition trials

At the start of each trial, the pumps were activated and the card barriers removed so that the focal fish could see the stimulus fish. The trial started when the focal fish was in the no preference zone so that the fish's preference was not biased. The fish was then observed for a 10 min period and the time spent in each of the preference zones recorded. The focal fish had to enter both preference zones in at least three of the four trials for the experiment to be valid, which ensured that the fish took part in at least one of either the matched or mismatched trials. Thus, I controlled for any side biases, and made certain that the fish had investigated both preference zones in more than 50% of the trials. After each trial, the focal fish was removed and the experimental tank was cleaned thoroughly with 100% alcohol and rinsed with a powerful jet of water. This removed the chemical cues from the stimulus fish and therefore

prevented the focal fish becoming overly familiar with them. The tank was then refilled and the focal fish reintroduced. All fish were then fed either crushed dry food or frozen Daphnia and left to feed. Approximately 30 min later, all fish were removed from their tanks and placed into temporary holding tanks while both the experimental and stimulus tanks were cleaned and refilled as before. This removed any traces of food that could have degraded water quality and otherwise affected the chemical cues. The fish were returned and given another 22 h acclimation period before the next trial. This protocol was followed until the fish had completed all four trials. Care was taken to ensure that the same silicon tubing was used for the same stimulus fish over all four trials. Between trials the tubing was flushed with clean water and then allowed to empty to remove chemical traces. Fresh tubing was used for each different focal individual that was tested. In total, 11 males and 13 females completed the experiment.

4.3.4 Assessing the effects of inbreeding on breeding preferences and breeding success

From stocks of virgin F1's of known parentage, 15 families were chosen for breeding. However, two fish died during the experiment (by jumping from tanks), so N = 13 families. Two breeding trials were carried out in a randomised order: In one trial, one male and one female sibling pair per family, which were unfamiliar to each other, were paired to breed. In the other trial, the original breeding pair was crossed with another unrelated sibling pair, so that the male was paired with an unfamiliar non-sibling female and the female with an unfamiliar non-sibling male. This cross-over breeding design meant that an individual's breeding propensity and success with a related and an unrelated individual could be compared. The sibling pairs used were chosen randomly.

Before the experiment, fish were anaesthetised, sexed and swabbed for microsatellite analysis. Relatedness scores could be calculated from this to assess if the degree of relatedness between pairs influenced their propensity to breed. Females were introduced into a 150 litre breeding tank (80 x 40 x 50 cm high) 24 hours before the male, to minimise aggression between the newly formed pairs. Two breeding shelters made from terracotta flower pots cut in

half were placed at the bottom of each tank. Refuge tubes made from clear plastic tubing were positioned at the top of the tank, allowing fish that received aggression from their partner to shelter away from them. Fish were given a maximum of six weeks to breed (as naïve breeders, *N. pulcher* were found to take longer to breed than the experienced adult stock breeders). Breeding shelters were checked each morning for eggs. The number of eggs was counted and any white eggs were carefully removed from the clutch to prevent disease spread. The shelter and eggs were then placed in a breeding net suspended within the parent's tank with an airstone bubbling over the eggs to keep them aerated. On days 2 and 3 after laying, the number of eggs was counted again and any diseased eggs removed. By counting eggs over three consecutive days we gained a more accurate representation of hatching success, as eggs are usually all hatched 4 days after laying and any un-hatched eggs are easy to see and count. The date on which eggs were laid was also noted and the number of days pairs took to breed calculated.

After the first breeding round was complete, all remaining shelters were removed from the breeding tanks to prevent re-breeding. Breeders were then rested for at least two weeks. Fish were re-sexed to identify them within their pair, measured and re-paired (as above). The second breeding trial then commenced. Of the 13 pairs used in the cross-over breeding design, 9 males and 8 females bred at least once with a sibling or non-sibling.

4.3.5 Assessing relatedness of original breeders and F1 pairs

DNA samples were collected from all of the original breeding stock of adults, as well as from their offspring that were used in the kin recognition and inbreeding experiments. The DNA collected from the stock population was used to ensure that they were not inbred. Further, the DNA from the offspring was used to assess if maximum likelihood relatedness scores between individuals had any influence on kin recognition or breeding. DNA was collected either as a fin clip or by swabbing. These two different methods of DNA collection were used to carry out a validation study of swabs as a less invasive method of collecting DNA samples from fish (see Le Vin et al., 2010b and Appendix II). Fin clips were taken from the caudal fin of each fish (approx 5 mm) and swabbing was carried

out by running a Barloworld Scientific sterile rayon tipped swab six times down the length of the body of the fish (Alison Bell, personal communication). Both fin clips and swabs were stored in 100% alcohol at 4°C prior to DNA extraction. Fin clips were collected at the end of breeding or experiments, in case altering the phenotype of an individual altered their behaviour. The specific method of DNA collection used in each part of the experiment is clarified within the relevant methodology sections below.

DNA from fin clips was extracted using the DNeasy Blood and Tissue Kit (QIAGEN). DNA from swabs was extracted using an extraction protocol for swabs in the QIAamp DNA Micro Handbook (QIAGEN), using QIAshredder spin columns (QIAGEN) and the DNeasy Blood and Tissue Kit (QIAGEN). Individuals were then genotyped at up to eight microsatellite loci (see Table 4-2). The forward primer of each pair was labelled with the ABI fluorescent dyes NED (yellow), HEX (green) and 6-FAM (blue) (see Table 4-2). Products were amplified by multiplex PCR, using the default reagent concentrations recommended by the kit instruction manual (Qiagen Inc, Crawley, UK). Two multiplex PCR's, one at 53°C and the other at 60°C, were carried out. Thermocycling was performed on a DNA Engine Dyad (MJ research, Waltham, MA) using the following program: initial denaturation for 15 min at 95°C, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at either 53°C for 60 s or 60°C for 90 s and extension at 72°C for 90 s, followed by a final 10 min extension at 72°C. Multiplexed products (1:160 dilutions) were genotyped using an ABI 3730 sequencer (by The Sequencing Service, University of Dundee, UK). Genotypes were read, corrected by eye and analysed using GeneMapper version 4.0 (Applied Biosystems, Foster City, CA); ROX GS400HD was used as the size standard ladder. Relatedness between individuals was then calculated using the programme ML-Relate (Kalinowski et al., 2006), which calculates maximum likelihood estimates of relatedness based on allele sharing.

4.3.6 Data Analysis

All data were analysed using SPSS version 10.0.5 (SPSS Inc., Chicago, IL U.S.A.). Data were checked for normality and homogeneity of variance. Where data and residuals were not normal, non-parametric tests were carried out. All tests were

two tailed and means and standard errors are presented throughout. For the adult kin recognition experiments comparing mean time spent with unfamiliar kin versus unfamiliar non-kin, paired t-tests were carried out. To tease out whether chemical or visual cues were most important, we compared the time spent with kin in the matched-cues test (chemical and visual cues from kin matched), with the time spent with either the chemical or visual cues of kin during the mismatched-cues test. For the inbreeding experiment, a Mann Whitney U-test was carried out to assess if relatedness affected propensity to breed. Pearson's correlations were used to check for relationships between SL and weight in males and females, and to assess whether male or female SL was correlated with the total number of eggs laid or the proportion of eggs hatching. Paired t-tests were carried out to investigate if relatedness affected latency to breed, or breeding success. Individuals that did not breed were given a latency of 42 days, the maximum time allowed to breed. Finally, a Mann Whitney U-test was carried out to further assess the effect of relatedness on breeding success.

Table 4-1. Correlations between measurements taken on facial stripes of both males and females. Each individual has two stripes and each had length, breadth and area measured. The table shows that the area of stripe 1 was positively correlated with the area of stripe 2. This was also true for the length and the breadth. Further the table shows positive correlations between the mean area of the two stripes and the mean length and breadth. As the mean area of the facial stripes was correlated highly with all other measurements it was used in all further analysis.

Male	**Pearson's *r***	N	P
Area of stripe 1 & 2	0.77	33	<0.001
Length of stripe 1 & 2	0.68	33	<0.001
Breadth of stripe 1 & 2	0.78	33	<0.001
Mean area and mean length	0.90	33	<0.001
Mean area and mean breadth	0.90	33	<0.001

Female	**Pearson's *r***	N	P
Area of stripe 1 & 2	0.81	39	<0.001
Length of stripe 1 & 2	0.75	39	<0.001
Breadth of stripe 1 & 2	0.55	39	<0.001
Mean area and mean length	0.91	39	<0.001
Mean area and mean breadth	0.91	39	<0.001

Table 4-2. Microsatellite loci used for genotyping *Neolamprologus pulcher*, showing size range of each microsatellite, variability, the primer sequence, the two different annealing temperatures (T_m) used in the PCR, the dye label used and the original source of the microsatellite. The size standard ladder used was ROX GS400HD.

Primer	Size range	Alleles/unrelated individuals tested	Primer sequence (5' – 3')	T_m	Dye	Source
UNH106	133 - 137	3 / 39	5' - CCT TCA GCA TCC GTA TAT - 3' 5' - GTC TCT TTC TCT CTG TCA CAA G - 3'	53˚C	6-FAM	(Lee & Kocher, 1996)
NP773PT	136 -167	11 / 128	5' - ATC AGC ACG TCA TCT GCA TGA G - 3' 5' - GTT TCT TGC AAA GCA AAG CTG AGA AAC AA - 3'	60˚C	NED	(Schliewen et al., 2001)
ULI2PT	154 - 232	17 / 128	5' - TAA GTT CCA TGC ACC GAG ATA 5' -GTT TCT TTA TGG GAA CCT GTG AAT GTG AG -3'	53˚C	6-FAM	(Schliewen et al., 2001)
UME003PT	200 - 273	18 / 128	5' - GCC ACA TGT AAT CAT CTA ACT GC - 3' 5' - GTT TCT TGA GAT TTT TTT TGG TTC CGT TG - 3'	53˚C	HEX	(Parker & Kornfield, 1996)
TmoM11PT	177 - 232	16 / 125	5' - ATT CAG GTA GAG ACG AAA TAT TA -3' 5' - GTT TCT TTA GTC ACA GTT TAC ACA CAA C -3'	53˚C	NED	(Zardoya et al., 1996)
TmoM13PT	240 - 288	9 / 16	5'-CGC AGG GTG TTC TTC AGG TGT AT-3' 5'-GTT TCT TAA ATC ACC ATA TTC ATA TGT T-3'	53˚C	6-FAM	(Zardoya et al., 1996)
NP007PT	274 - 299	6 / 128	5' - TCA GAG TGC AAT GAG ACA TGA - 3' 5' - GTT TCT TAA TTT AGA AGC AGA AAA TTA GAC G - 3'	60˚C	HEX	(Schliewen et al., 2001)
TmoM27PT	385 -389	2/39	5' – AGG CAG GCA ATT ACC TTG ATG TT - 3' 5' – GTT TCT TTA CTA ACT CTG AAA GAA CCT GTG AT - 3'	60˚C	HEX	(Zardoya et al., 1996)

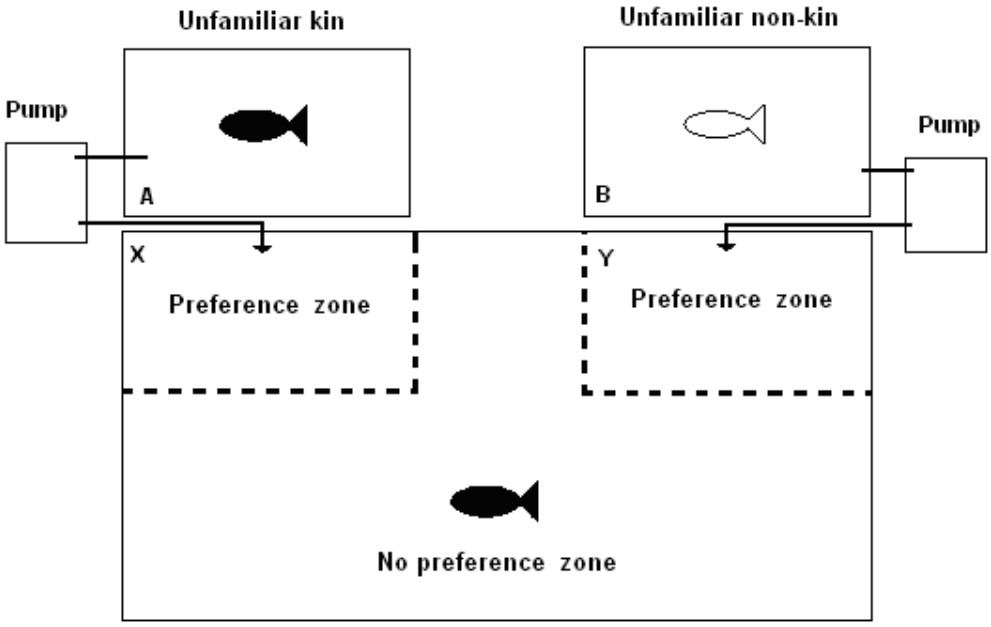

Figure 4-1. Schematic of experimental set-up for inbreeding avoidance experiments. Dashed lines indicate the two preference zones in the experimental fish tank. In the matched-cues test, the pumps carried stimulus water from tank A to preference zone X and from tank B to preference zone Y, as shown. In the mismatched-cues test the pumps carried stimulus water from tank A to preference zone Y and tank B to preference zone X. Diagram not to scale.

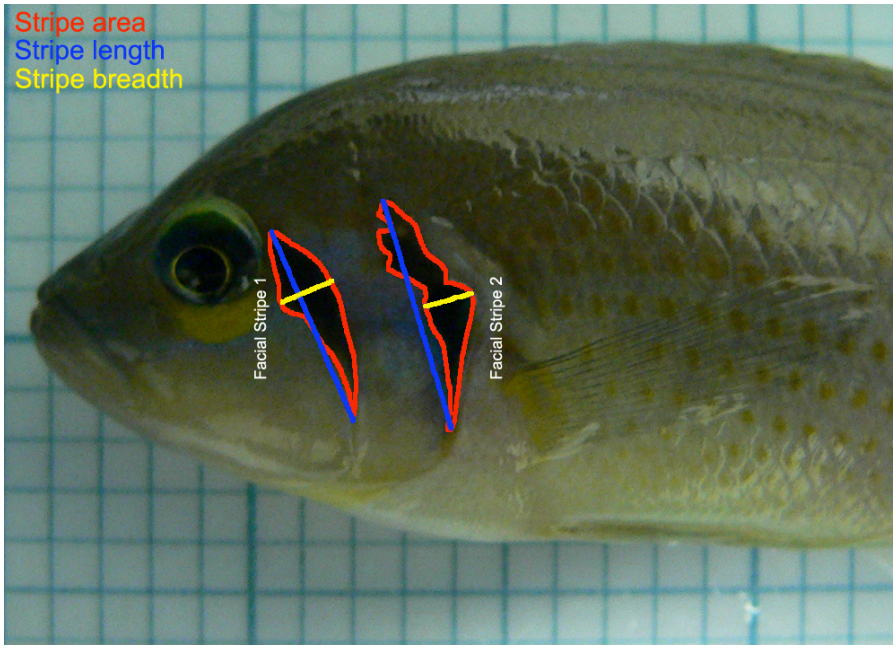

Figure 4-2. Figure showing measurements taken for facial stripe analysis. Three separate measurements were taken as indicated by the coloured lines: area (red), length (blue) and breadth (yellow) of each of the two stripes.

4.4 Results

4.4.1 Relatedness of breeding stock fish

All stock fish used as breeders were successfully genotyped at a minimum of seven loci and the average number typed per individual was 7.98. One individual at one locus (UME003PT) did not amplify. Overall, relatedness between the breeders was low, with only 5 of the 56 pairs having a relatedness score above that of cousins (r = 0.125) and 41 of the pairs having a relatedness score of zero. Although there was little evidence of homozygous excess (Table 4-3), at TmoM13PT for the captive-bred and TmoM11PT for the wild-caught, the observed heterozygosity was lower than that of the expected. Further, locus TmoM27PT had very low heterozygosity across both populations. However, overall, genetic diversity was high in our breeding stock and there is no evidence of inbreeding in either stock population. Relatedness scores between pairs did not affect the propensity to breed or not to breed (Mann Whitney U test; U = 354, N = 56, P = 0.44). Some individuals never bred and so may have been infertile. However, even when they were removed from the analysis, relatedness did not affect propensity to breed (Mann Whitney U test; U = 111, N = 37, P = 0.88). Relatedness scores between breeding pairs from the two populations were not found to differ significantly (Mann Whitney U test; U = 243, N = 56, P = 0.11). However, the fry survival of the wild-caught population was found to be significantly greater than the fry survival of the captive-bred population (Mann Whitney U test; U = 22, N = 29, P < 0.001). Overall, relatedness between individuals in the adult breeding stock was very low, so the F1's to be used in the experiments should have been genetically diverse.

4.4.2 Kin recognition of opposite sex adults

1) Do sexually mature *N. pulcher* show preferences for associating with kin or non-kin of the opposite sex and were chemical and/or visual cues more important in their preferences?

In the matched cues test, male *N. pulcher* did not show any preference for associating with either unfamiliar female non-kin or unfamiliar female kin, when

presented with both chemical and visual cues (Paired t-test; t_{11} = 0.8, P = 0.45; Figure 4-3a).

In the matched cues test, female *N. pulcher* showed preferences for associating with unfamiliar male kin over unfamiliar male non-kin, when presented with both chemical and visual cues (Paired t-test; t_{13} = 2.15, P = 0.05; Figure 4-3b). Females did not spend significantly longer with male kin when chemical and visual cues were matched than when they had visual contact with male kin in the mismatched-cues test (Paired t-test; t_{13} = 0.32, P = 0.75; Figure 4-3c). Females also did not spend significantly longer with male kin when chemical and visual cues were matched than when they had chemical contact with male kin in the mismatched-cues test (Paired t-test; t_{13} = 0.33, P = 0.75; Figure 4-3d). Taken together, these results show that both visual and chemical cues are important in a female's choice of which male to associate with.

2) Do relatedness scores between the experimental and stimulus fish correlate with the strength of their preference?

There was no correlation between the difference in time focal males spent with the stimulus females (mean time spent with kin – mean time spent with non-kin) and the difference in relatedness score (relatedness score to kin – relatedness score to non-kin) (Pearson's correlation; r = -0.16, N = 11, P = 0.65; Figure 4-4a). Similarly, there was no correlation between the difference in time a female spent with the stimulus males and the difference in relatedness score (Pearson's correlation; r = 0.26, N = 13, P = 0.28; Figure 4-4b). Further, although females preferred to associate with male kin, they did not spend more time with them as their relatedness score to them increased (Pearson's correlation; r = 0.32, N = 13, P = 0.28; Figure 4-5).

3) Do the facial stripe size of stimulus fish influence mate preference?

In the male experiment, mean stripe area did not differ significantly between the kin and non-kin stimulus females (Paired t-test; t = -0.54, N = 11, P = 0.60). Similarly in the female experiment, mean stripe area did not differ between the stimulus male kin and non-kin groups (Paired t-test; t = -0.28, N = 13, P = 0.78). There was no correlation between the difference in time the males spent with a

stimulus female in the matched cues experiment (time spent with preferred female – time spent with non-preferred female) and the difference in female's mean stripe area (stripe area of preferred female – stripe area of non-preferred female) (Pearson's correlation; r = 0.40, N = 11, P = 0.22). This was also true for the female experiment (Pearson's correlation; r = -0.07, N = 13, P = 0.82). So, males and females showed no preferences for associating with opposite sex individuals dependent upon the size of their facial stripes.

4.4.3 Assessing the effects of inbreeding on breeding preferences and/or breeding success

4) Do relatedness scores between breeding pairs affect willingness to breed?

Pairs that bred did not have significantly different maximum likelihood relatedness scores from pairs that didn't breed (Mann Whitney U test; U = 95.5, N = 28, P = 0.93; Figure 4-6).

5) Is breeding success lower in sib-sib pairs compared to non-sib pairs?

Male SL did not correlate with the total number of eggs laid (Pearson's correlation; r = -0.06, N = 18, P = 0.85) or the proportion of eggs hatching (Pearson's correlation; r = -0.14, N = 18, P = 0.57). Clutches were not larger when males bred with a related female versus an unrelated female (Paired t-test; t = -0.24, N = 9, P = 0.82). Males also did not breed more quickly with a related versus an unrelated female (Paired t-test; t = -1.71, N = 9, P = 0.13). For males that bred at least once, there was a non-significant trend for males breeding with related females to have a greater proportion of eggs hatching than males that bred with unrelated females (Paired t-test; t = 2.04, N = 9, P = 0.08; Figure 4-7a).

Female SL was correlated with total number of eggs laid (Pearson's correlation; r = 0.77, N = 15, P < 0.001), but SL was not correlated with hatching success (Pearson's correlation; r = -0.08, N = 16, P = 0.78). Females did not lay more eggs when bred with a related versus an unrelated male (Paired t-test; t = -0.41, N = 8, P = 0.69), nor did they breed more quickly with a related over an unrelated male (Paired t-test; t = -1.2, N = 8, P = 0.27). Of the females that

bred at least once, there was a non-significant trend for females breeding with a related male to have a greater proportion of eggs hatching (Paired t-test; t = 2.15, N = 8, P = 0.07; Figure 4-7b) than females breeding with an unrelated male.

When investigating hatching success, the paired analysis presented above, included individuals that had bred just once, with either a related or an unrelated partner, as well as individuals that had bred with both. Therefore, for some individuals, a hatching success of zero was recorded. However, this may be misleading, as rather than having no eggs hatching, some individuals just hadn't bred. Therefore, when looking only at individuals that did breed and not using paired analysis, I found a non-significant trend for sibling pairs to have increased hatching success over non-sibling pairs (Mann Whitney U test; U = 11.0, N = 15, P = 0.054; Figure 4-8).

Table 4-3. Observed (Ho) and Expected (He) heterozygosity of each microsatellite for the two populations of original breeders used to create the sibling and non-sibling groups, and mean relatedness of populations (r ± S.E.).

Population	Microsatellite locus	H_o	H_e	r
Captive-bred	TmoM11PT	0.91	0.89	0.06 ± 0.03
	TmoM13PT	0.67	0.82	
	ULI2PT	0.82	0.89	
	UME003PT	0.91	0.90	
	UNH106	0.61	0.54	
	NP007PT	0.52	0.53	
	NP773PT	0.85	0.81	
	TmoM27PT	0.12	0.12	
Wild-caught	TmoM11PT	0.87	0.95	0.02 ± 0.01
	TmoM13PT	0.94	0.95	
	ULI2PT	0.97	0.94	
	UME003PT	0.94	0.93	
	UNH106	0.45	0.41	
	NP007PT	0.58	0.55	
	NP773PT	0.81	0.84	
	TmoM27PT	0.00	0.00	

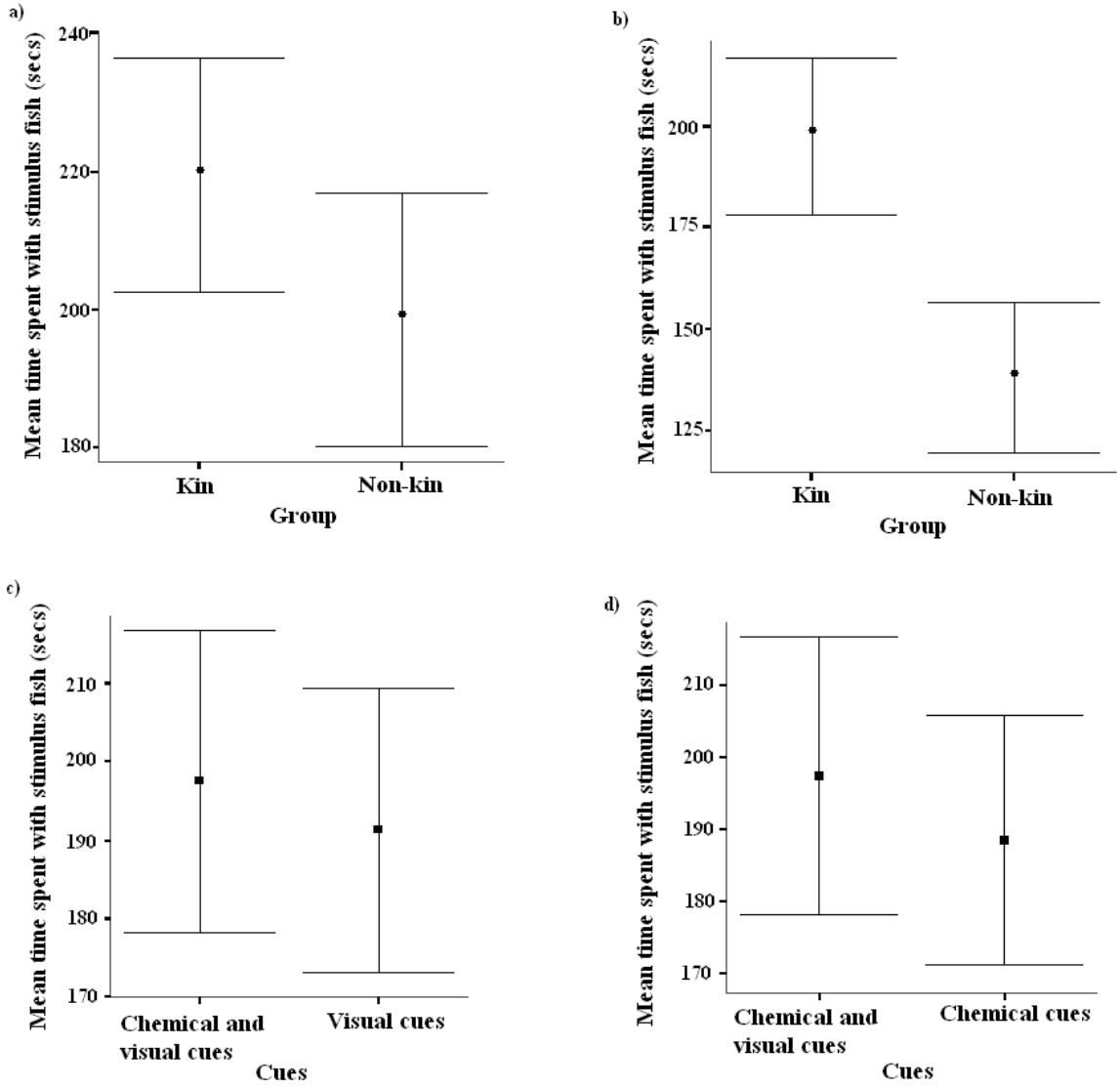

Figure 4-3. Time spent associating with opposite sex kin or non-kin in adult kin recognition experiments. In the matched-cues tests: a) males did not discriminate between sisters and non-kin (P = 0.45); b) females spent more time with brothers over non-kin males (P = 0.05). In the mis-matched cues test, females: c) spent equal amounts of time with either both the chemical and visual cues, or just the visual cues of brothers (P = 0.75); and d) spent equal amounts of time with either both chemical and visual cues, or just chemical cues of brothers (P = 0.75). Error bars show mean time ± S.E.

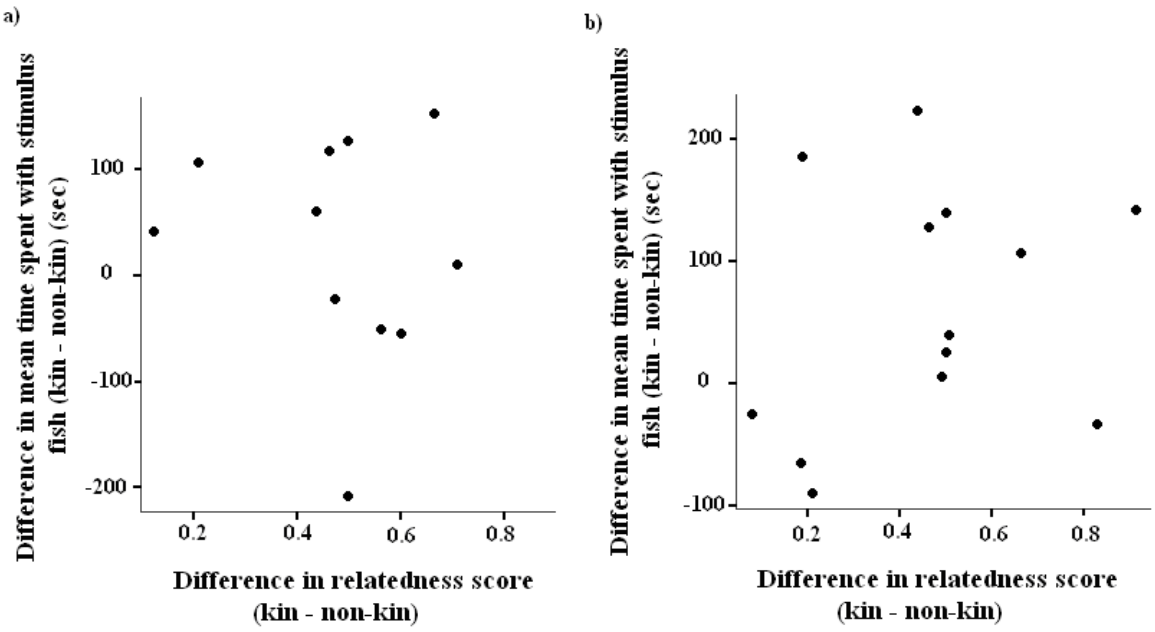

Figure 4-4. Lack of correlations between the difference in time (secs) spent with the stimulus fish (kin – non-kin) and the difference in relatedness scores between the focal and stimulus fish (kin – non-kin) for: a) the male matched cues kin recognition experiment (P = 0.65); and b) the female matched cues kin recognition experiment (P = 0.28).

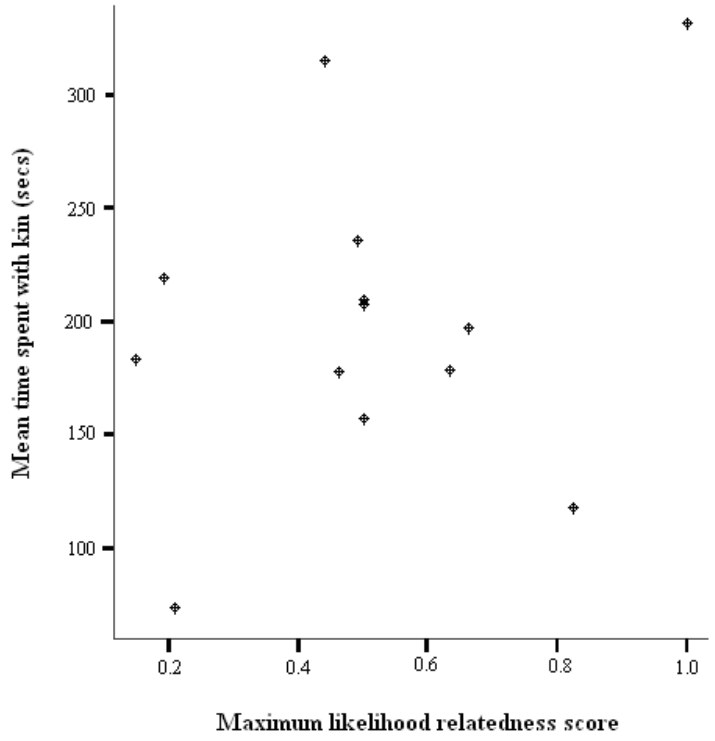

Figure 4-5. Female matched-cues kin recognition experiment. Lack of correlation between the mean time spent with male kin and the maximum likelihood relatedness score of the female to the male kin (P = 0.28).

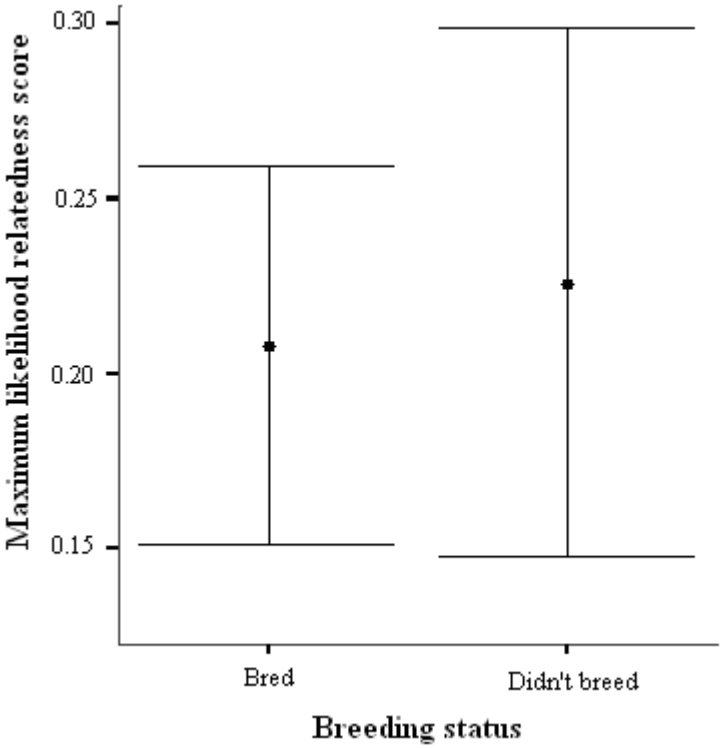

Figure 4-6. Relatedness scores between pairs and their propensity to breed in the inbreeding experiment. Relatedness to a breeding partner did not affect propensity to breed (P = 0.94). Error bars show the mean maximum likelihood pairwise relatedness ± S.E.

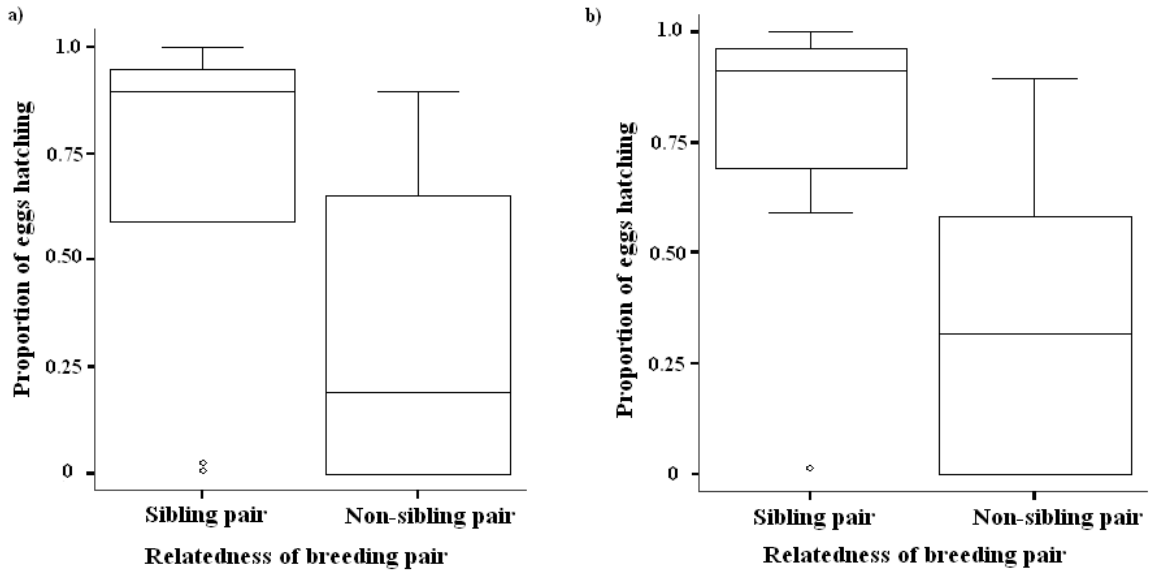

Figure 4-7. Graphs showing the proportion of eggs hatching for sibling and non-sibling pairs. Although there was not a significant difference in the means, the proportion of eggs hatching between sibling pairs tended to be greater than that of non-sibling pairs in both: a) male (P= 0.08); and b) female breeders (P= 0.07). On the boxes, horizontal lines represents the mean amount of fry hatching, the top and bottom of the box the 75th and 25th percentiles, the whiskers largest values which were not outliers, and the circles are extreme outliers.

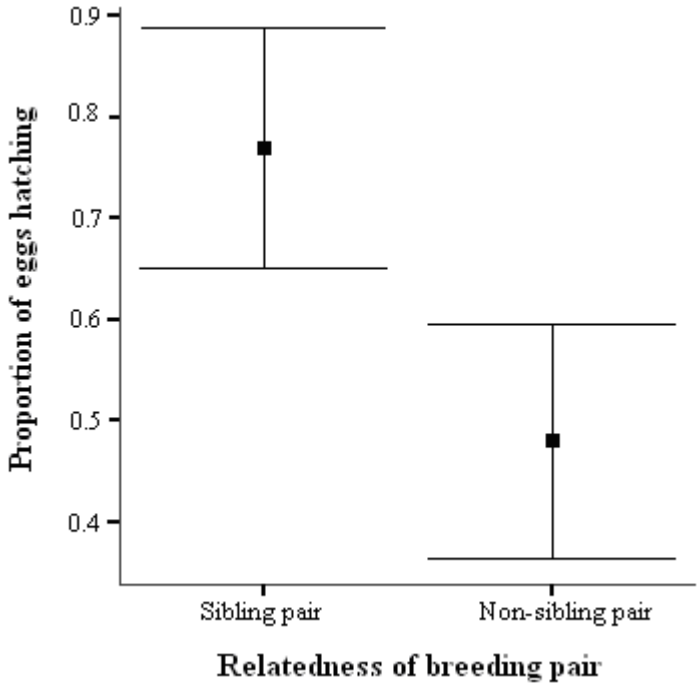

Figure 4-8. Graph showing the proportion of eggs hatching for sibling and non-sibling pairs, including only those pairs that actually bred. Non-significant trend for sibling pairs to have increased hatching success over non-sibling pairs (P=0.054). Error bars show the proportion of eggs that hatched ± S.E.

4.5 Discussion

In this study, I found evidence that, in the absence of familiarity, sexually mature female *N. pulcher* show preferences, using both visual and chemical cues, for kin over non-kin males. In contrast, males showed no preference for either kin or non-kin females. Thus, my results show that females recognise and discriminate between kin over non-kin. Males, in contrast, did not discriminate between kin or non-kin, although it is likely that they can recognise them, as previous work has found that juvenile *N. pulcher* can recognise kin (see chapter 2 and Le Vin et al., 2010a). Relatedness scores, based on the proportion of alleles shared between individuals, were not found to correlate with the observed variation in association preferences. In addition, *N. pulcher* showed no preference for individuals based on the size of their facial stripes. So, facial stripes size does not appear to be used for association preferences in *N. pulcher*, although other attributes of facial stripes such as colour could be important for individual or mate preferences. Microsatellite-based analyses also confirmed that the parental breeding stock were not inbred, so preferences for kin or non-kin were not confounded by them being closely related. Further, I found that relatedness to a breeding partner had no effect on their propensity to breed. However, in both males and females, there was a trend for sibling pairs to have increased hatching success, compared to unrelated pairs. So, in *N. pulcher* breeding appears to be random, although breeding with relatives may bring some benefits over breeding with non-relatives.

To avoid inbreeding, it would be expected that adult *N. pulcher* would prefer to associate with non-kin over kin of the opposite sex. In contrast, male *N. pulcher* in this study were found to show no preference for kin over non-kin, and females preferred kin. This corresponds with Stiver et al (2008), who found that *N. pulcher* bred randomly in the wild, neither actively seeking out, nor avoiding genetically similar individuals as mates. In other fish species, such as zebrafish, *Danio rerio,* males also have been found to show no preference for kin or non-kin females (Gerlach & Lysiak, 2006). So, males are likely to be trying to maximise their breeding opportunities, and hence fitness, by associating with as many females as possible (Trivers, 1972). Male *N. pulcher* are often polygynous (Limberger, 1983), and unlike females, must compete rather than inherit

territories (Stiver et al., 2006); they also tend to have a shorter tenure on territories than females (Stiver et al., 2004). In such circumstances, males may not be able to afford to be discriminatory, and simply mate with as many females as possible, regardless of relatedness. Female *N. pulcher,* on the other hand, preferred male kin over non-kin. Females are generally assumed to be the choosier sex, selecting the highest quality mate to maximise their fitness (Trivers, 1972). Studies have shown in other species that females often avoid male kin as potential mates, for example, rainbowfish, *Melanotaenia eachamensis*, (Arnold, 2000) and house mice, *Mus musculus*, (Winn & Vestal, 1986). However, our study also found that inbreeding in this species may not necessarily be deleterious. Since female *N. pulcher* in the wild may inherit their natal territory (Stiver et al., 2006), a female may have to breed with the dominant male regardless of her relatedness to him, or alternatively, give up her valuable breeding spot. Furthermore, as there is evidence of extra pair paternity in *N. pulcher*, with extra pair males siring up to 22.9% of a clutch (Dierkes et al., 1999; Dierkes et al., 2008; Heg et al., 2006), females may breed with multiple males of differing relatedness. Therefore, inbreeding may be less of an active choice and more of a consequence of the life history of this species. Combined with the fact that *N. pulcher* are as likely to breed with kin as with non-kin, my findings support those of Stiver et al's (2008), that *N. pulcher* breed at random with respect to allele sharing between pairs, regardless of whether these alleles are identical by descent or not.

Sibling pairs showed a trend to have increased hatching success compared to pairs of non-siblings (Figure 4-7 and Figure 4-8). Consequently, it would appear in *N. pulcher* that inbreeding may not be disadvantageous. Although our sample size was small in this analysis, inbreeding has been found to be beneficial in other species. For example, in the cichlid *P. taeniatus* (Thunken et al., 2007), males and females preferred to associate with and mate with opposite sex siblings over non-siblings. Also, related pairs showed better parental care. Furthermore, inbreeding in Ambrosia beetles, *Xylosandrus germanus,* like *N. pulcher,* also increased hatching success (Peer & Taborsky, 2005). Although inbreeding may not de detrimental in *N. pulcher*, I found that individuals were just as likely to breed with related as unrelated partners. It is possible that there is a trade-off in breeding strategies, with inbreeding favoured under some

conditions and outbreeding under others. Apparently opportunistic breeding in *N. pulcher* could allow bet-hedging to optimise fitness (Philippi & Seger, 1989; Slatkin, 1974), by mating with a relative rather than forgoing breeding altogether. Alternatively, age or condition may influence when individuals are more receptive to inbreeding. For example, Richard et al (2009) found that in the common lizard, *Lacerta vivipara,* intermediate aged females, which are of the best quality, do not breed with genetically similar males. On the other hand, young and old females, which are of a lower quality than the intermediate females, will breed with genetically similar males. In my study, the fish, although of a sexually mature size, were naïve breeders. So, it is possible that more experienced individuals would be choosier with respect to inbreeding compared to those of inexperienced ones. Overall, whilst our study may have found benefits to breeding with relatives, it seems likely that in *N. pulcher* inbreeding may be a trade-off to the alternative of not breeding at all. Further studies are needed to ascertain what other factors may influence inbreeding in this species.

Constraints on independent breeding, attaining breeding positions and dispersal may go some way to explain the random breeding structure of *N. pulcher*. Laboratory experiments have shown that helpers, given the chance, will disperse to new breeding territories (Bergmüller et al., 2005b). However, in the field, it has been found that although suitable breeding territories are available around existing colonies (made up of 2 – 200 breeding territories (Balshine et al., 2001), helpers prefer to take up residence in a territory situated within, rather than on the edge of an existing colony (Heg et al., 2008a). Presumably, this is because it offers them more protection from predators. Hence, individuals are particular about where they will breed, preferring to wait for a good quality territory (Heg et al., 2008a), than make do with a poor one. Individuals are also generally more related to their own group members than to conspecifics on other territories (Stiver et al., 2007). However, relatedness of helpers to breeders in groups decreases with helper size and thus age (Dierkes et al., 2005). In this study, looking at the diversity of the two original populations of stock breeders used to create the sibling groups, we can see that both the wild-caught adults and the captive-bred population showed very low levels of relatedness (wild-caught population; Mean = 0.02 ± 0.01; captive-bred population; Mean = 0.06 ± 0.03). In

addition, there was no evidence of there being an excess of homozygotes, and thus, there is no evidence of inbreeding in these populations. So, overall relatedness between individuals within a group of *N. pulcher* is not likely to be particularly high. Finally, dispersal, although it is limited (see Heg et al., 2005; Stiver et al., 2004) is generally sex-biased, with large males dispersing the furthest (Stiver et al., 2004; 2006). This, coupled with a lack of breeding opportunities and generally low relatedness amongst group members, may reduce the chances of *N. pulcher* actively inbreeding with closely related relatives in the wild, regardless of whether inbreeding has potential fitness advantages.

Kokko and Ots (2006) suggest that inbreeding should be tolerated more when breeding opportunities are sequential rather than simultaneous. This is because an individual does not know when, or if, another partner will come along. Therefore, inbreeding is a better option than not breeding at all. In my experiment, individuals had sequential breeding opportunities, so it is possible that our lack of inbreeding avoidance may have just been an artefact of the experimental design. However, Stiver et al (2008) reports cases where even when presented simultaneously with mates differing in relatedness and familiarity, *N. pulcher* showed no preferences. Furthermore, in *N. pulcher* mating generally is sequential. Therefore, overall evidence suggests that the inbreeding observed in my experiment reflects natural behaviour, and that *N. pulcher* in the wild and in captivity will breed as readily with relatives as non-relatives.

Hatching success is often used to measure the effects of inbreeding (Mrakov & Haley, 1979; Spottiswoode & Møller, 2004; Su et al., 1996), as it gives a measure of fertility. However, the effects of inbreeding on an individual may not become obvious until later in their life. For example, inbred offspring may have reduced growth, delayed sexual maturity, reduced fertility or increased mortality (reviewed for salmonids in Wang et al., 2001). Alternatively, the deleterious consequences of inbreeding may only become apparent after several generations of related pairs breeding. For example, convict cichlids, *Cichlasoma nigrofasciatum,* only show deformities after 4 to 5 generations of inbreeding (Winemiller & Taylor, 1982). Thus, investigating hatching success may not expose the full consequences of inbreeding. Consequently, further investigation,

to assess the potential long-term and inter-generational effects of inbreeding in this species are required.

Facial stripe size was not found to influence the preference of *N. pulcher* for associating with individuals of the opposite sex. In other species, phenotypic traits can be sexually selected for, for example, male swordtails, *Xiphophorus hellerii*, with longer swords are preferred by females (Basolo, 1990). However, it seems in *N. pulcher* that facial stripe size does not influence mate choice or recognising kin (although we do not know if it influences dominance). However, it is possible that other features of the stripes may influence preference, such as colour intensity. Individuals vary greatly in the colours surrounding their facial stripes and eyes (Le Vin, personal observations). Here, individuals may have blue, yellow, purple and/or turquoise colouration. Other studies investigating colour have found mate choice for brighter individuals (Bakker & Mundwiler, 1994; Hill, 1990). Thus, it would be interesting to further investigate if the colours exhibited by *N. pulcher* have some role in mate choice and/or in kin recognition. Experiments involving manipulation of the facial stripes would give more conclusive results than simply examining natural variation.

In conclusion, in captivity *N. pulcher* do not actively avoid associating with or inbreeding with kin. Further, hatching success is somewhat increased in sibling breeding pairs, compared to non-sibling pairs. Therefore, in this species inbreeding may not be deleterious. However, as individuals do not more readily breed with kin over non-kin, it would suggest that although inbreeding can be tolerated it is not necessarily the norm. Females in the wild are unlikely to be able to exert control over which male acquires their breeding territory and males also may be limited in which territories they can overtake. Further, due to dispersal by males and territory inheritance by females, relatedness between individuals within a territory is not likely to be high. Consequently, although inbreeding may bring some advantages, the life history of the species means the chances of it occurring may be quite rare. It remains to be tested, however, whether breeding between close relatives has longer-term impacts on the fitness of both breeders and their offspring.

Chapter 5: Male mate choice in the green swordtail *Xiphophorus hellerii*: the importance of visual and chemical cues

5.1 Abstract

Females are generally the choosier sex, but males should also be choosy under certain circumstances, e.g. when females vary in quality. Using variants of two-way choice tests, I investigated whether male green swordtails *Xiphophorus hellerii* preferred large or small females and the cues used in mate choice. Males preferred the large over the small female when visual, but not chemical, cues were available, even though females were non-responsive to male courtship. However, as the relative difference in size between the large and small female widened, the relative strength of male preference for large females increased significantly with chemical cues and marginally so with visual cues. Overall, visual cues elicited stronger male mate preferences than chemical cues, which only stimulated males to prefer large females over a certain size differential. In a species living in clear tropical streams, the bias towards using visual cues during mate selection makes ecological sense. Chemical cues may provide a secondary source of information for males, particularly when waters become seasonally turbid. Yet to be discovered is whether a male's selection of large females enhances fitness via increased fecundity and/or reduced costs of mate detection and assessment in terms of time, energy or predation risk.

5.2 Introduction

As eggs are generally more costly to produce than sperm, females tend to be the choosier sex, whilst males compete for access to females. Thus, males are predicted to maximise their fitness by reproducing with as many females as possible, whilst females should select males of high quality (Trivers, 1972). Many studies have shown that females benefit from being choosy either directly through, for example, selecting a mate who provides good parental care (Lindström et al., 2006), a good territory (Searcy, 1979) or nuptial gifts (Reinhold, 1999), or indirectly, by choosing a mate who provides good genes that will improve the fitness of their offspring (Doty & Welch, 2001). Less well studied is male mate choice. Anderson (1994) argued that males in certain situations should also be choosy; when females differ in their quality (e.g. size, fecundity or parental abilities), when males have access to a selection of females to choose from and/or where mating with one female reduces sperm reserves and hence their chances of fertilizing subsequent females. In these situations, males should be predicted to try and mate with the highest quality female so that they gain the highest fitness return. Many fish species have continuous growth throughout life, and female fecundity has been found to increase with female size (Bagenal & Braum, 1968; Cheong et al., 1984; Kraak & Bakker, 1998; Plath et al., 2006). Therefore, large females should produce more or larger eggs and/or more or larger fry than small females, and consequently be more attractive to males. Moreover, larger females are usually older than smaller females, so body size also demonstrates the ability to survive, another aspect of fitness. Accordingly, male mate preference for larger females has already been found in several species of fish (Côte & Hunte, 1989; Herdman et al., 2004). However, there may be different costs to males in choosing a large over a small females depending upon the size of the male. Larger males may have increased mating success (Côte & Hunte, 1989), and often females prefer larger over smaller males (Basolo 1998; Rosenthal and Evans 1998). Thus smaller males may have to work harder to court a female or could be outcompeted by a larger male. However, sometimes in mate choice studies, the role of chemical and visual cues in mate choice are not separated (Deaton, 2008; Tudor & Morris, 2009; although see exceptions in Plath et al., 2006; Wong et al., 2005) or only visual cues are assessed (Basolo, 2004; Sargent et al., 1986; Werner & Lotem,

2003; Wong & Jennions, 2003). As visual and chemical cues may convey different information concerning the fitness of potential mates to individuals it is important to investigate the role of both in mate choice.

Visual cues have been found to be important in courtship and mate preferences in fish. For example, male three-spine sticklebacks, *Gasterosteus aculeatus*, will court large females more often than small females, even when chemical cues are occluded (Sargent et al., 1986). Similarly, male swordtails have also been found to show mate choice preferences based on visual cues. Benson (2007) found that male green swordtails, *Xiphophorus hellerii*, courted females with artificially enlarged gravid patches more often than females without altered brood patches. The gravid patch is a dark spot on the lateral aspect of the female's abdomen and is larger and more visible when a female is carrying more or larger eggs, and thus indicates a female's reproductive potential. So, visual cues may be used to infer body size and fecundity of mates. However, in a natural environment visual cues may be obscured, for example in turbid water (Heubel & Schlupp, 2006), so chemical cues may also be used to provide males with information of mate quality. Chemical cues in fish have been found to convey information about mates, such as nutritional state (Fisher & Rosenthal, 2006; Plath et al., 2005), heterozygosity at MHC loci (Landry et al., 2001) and relatedness (see chapter 2 and Le Vin et al., 2010a). Male swordtails of the species *X. birchmanni* (Lechner & Radda 1987) have also been found to use chemical cues to distinguish between conspecific and heterospecific females (Wong et al., 2005). However, few studies have fully separated visual from chemical cues when looking at male mate choice (although see Plath et al., 2006; Wong et al., 2005). Males may be able to assess female qualities such as size, either from the combination of volatiles in an individual's chemical 'signature', or simply by the volume or concentration of the female's chemical cues. Since chemicals are secreted through the skin or via urine, larger females may be predicted to produce more chemicals than smaller females. Both visual and chemical cues may be important mate choice, allowing males to assess mate qualities such as size and fecundity, thus it is important to assess them separately in order to fully understand which stimuli may be affecting mate choices.

5.2.1 Aims

My study aimed to investigate male mate choice in a Poeciliid fish species, the green swordtail, *Xiphophorus hellerii*, and to examine whether chemical or visual cues, or both, are used in mate choice. Swordtails are an ideal species for investigating male mate choice as female swordtails grow throughout life and hence vary a great deal in their body size (Basolo, 1990), and larger females have been found to give birth to heavier fry (Walling, 2006). Thus, there is potential that *X. hellerii* males may be choosy and prefer larger over smaller females. Swordtails are generally reported to live in clear shallow waters, so I would predict that visual cues should be particularly important in mate choice in this species. However, during the rainy season waters can become turbid (Franck et al., 1998) and in the dry season small pools often separate from the main stream and become murky (Personal communication, Martin Plath). Therefore, visual cues may be obscured meaning fish may need to be more reliant on chemical cues for mate recognition. I assess whether males show preferences for larger over smaller females based solely on their chemical cues or only their visual cues. I look at the effect of the female's relative body size on mate choice for the chemical cues test, and in addition, in the visual cues test I also look at gravid patch size, and assess male courtship displays towards the females. I hypothesise that male swordtails should prefer large females over small, and that visual cues may be more important than chemical cues in their preferences. Furthermore, I suggest that males, when given visual cues, should spend more time with females with larger gravid patches, as it may indicate female fecundity, and males should also show more courtship displays towards larger than smaller females.

5.3 Methods

All fish used in these experiments were sexually mature third and fourth generation offspring from wild-caught adult *X. hellerii*, from Belize. One month before the experiments began, groups of four to seven mature male swordtails (n = 17) from the stock population were randomly allocated to one of four 50 litre tanks (60 x 30 cm and 30 cm high). These tanks were divided in half by a clear plastic divider, allowing both visual and chemical transfer between the

compartments. A selection of large and small females was placed on the opposite side of the divide so that all focal males were exposed to a range of female body sizes, to standardize their experience of females. During the course of the behavioural mate choice tests, none of the females appeared to be pregnant and no fry were born. These females were also used as stimulus females in the behavioural mate choice tests. Tanks had a weekly 25% water change to maintain water quality and water temperatures were maintained at 24.05 ± 1.05°C, pH at 7-7.4, and light:dark regime of 13.5:10.5 hours. All water was charcoal filtered and aerated for at least 18 hours to reduce chlorine levels prior to use in tanks. Fish were fed once daily in the morning, on either commercial flake food or on frozen bloodworms. On experimental days, male fish were fed to satiation at least an hour prior to experimentation. This ensured that hunger levels were standardised and were not likely to affect the male's behaviour.

Between March and May 2007, two behavioural mate choice tests were carried out, with each test consisting of two phases. Swordtail males underwent a chemical preference test in which the male had to choose to associate with water containing the chemical cues from either a large or a small female. Males also underwent a visual preference test, where they had the choice of associating with only the visual cues of a large or a small female. As there were more males to be tested than there were pairs of large and small females, some females had to be used twice to either provide chemical cues or visual stimuli. However, care was taken that the same dyads of females were never presented twice and that the females were unfamiliar to the male. All tests were carried out between the hours of 10:00 and 15:30.

Association time was used as a measure of male preference in both the chemical and visual preference tests. Association time is often used as a standard assay of sexual response in Xiphophorus (Basolo, 1990; McLennan & Ryan, 2008), and in *X. hellerii* females it has been found that females, given a choice of males, are more likely to mate with the male which they spent longest with (Walling, 2006). Further, association time was a reliable predictor of male mate choice in the cichlid species *Pelvicachromis taeniatus* (Thünken et al., 2007). At the end of each chemical and visual preference test, the standard length (SL) (measured from the tip of the snout to the posterior end of the last vertebra), was recorded

for each fish. In the visual preference test, the width and height of the gravid patch on the females was also measured. At the end of all experiments all fish were then returned to their original tanks and maintained under the same tank conditions as previously described.

5.3.1 Chemical cues test

In the chemical cues test, males were allowed to choose between large and small females based only on chemical cues. By placing either one large female or one small female into a 4 litre tank, (height 25 x 15.5 cm x 15.5 cm high), provisioned only with an air-stone, for a period of at least 16 hours, water was imbued with their chemical cues. Females were fed at least 6 hours prior to being isolated, and were not fed during the isolation phase so as to prevent food odours from affecting their chemical signatures. Each female's experimental tank had a male swordtail in an adjacent tank to provide visual stimulus, as the chemical signals may be costly to produce and therefore individuals may only produce them when a member of the opposite sex is in close proximity (McLennan & Ryan, 1997). Large females (mean ± S.E. = 41.90 mm ± 0.67, range = 37.7 – 47.8 mm, N = 15) were significantly greater in SL than small females (mean± S.E. = 26.53 mm ± 0.78, range = 21.2 – 29.9 mm, N = 15; t-test; t = 14.92, N = 30, P < 0.001).

Male experimental tanks (60 x 30 cm x 30 cm high) were filled with 30 litres of water. The tank was divided into three zones: two 12 cm 'preference zones' at either end of the tank and a 36 cm 'no preference' zone in the middle (see Figure 5-1a). Zones were marked with black tape attached to the bottom of the tank. To prevent the possibility of disturbance during the trials all four sides of the tank were covered with thick paper and an angled mirror was placed above the tank so that the male could still be seen by the human observer.

A tank of water containing chemical cues from the large female was randomly positioned at one side of the male's tank and water from a small female was positioned at the other side. Silicone tubing ran from each of the tanks of stimulus water through a peristaltic pump (which controlled the rate of flow) to the male experimental tank. Here, the tubing was attached to a 15 cm glass pipette, which sat 1 cm above the water level of the male experimental tank

and above one of the preference zones. Pumps were set to a flow rate of 1.4 ml/min (my pump's lowest threshold), as previous work had found responses by Xiphophorus spp. to stimulus flows of 1.2 ml/min (McLennan & Ryan, 1997). Pilot studies with colour dyed water showed that the stimulus water from the pumps remained concentrated and mainly localized within the marked preference zones. One male was introduced to the experimental tank and after a 30 minute acclimation period, the pumps were started. Care was taken to ensure that the male was swimming freely in the centre of the 'no preference' zone when the pumps were started so that there was no bias to swim to a preference zone. The focal male then had a maximum of 10 minutes to explore both preference zones and come into contact with the chemical cues of the large and small female. This was termed the 'exploratory' period. Once the male had entered and fully exited both zones, a 5 minute observation period was started, regardless of how long into the 10 minute 'exploratory' period the male was. The amount of time the male spent in each preference zone during the 5 minute observation period was recorded, since this was considered to represent a choice to associate with that female's chemical cue. After this first phase, the male was removed and placed in a separate holding tank whilst the experimental tank was emptied. The tank was then thoroughly cleaned with 100% alcohol and rinsed well with a powerful jet of water, taking care to ensure the silicon seals were well washed and rinsed, before then being refilled. To account for any potential bias in tank side preference, the large and small female stimulus water tanks were switched and the male's preference tested again, following the same procedure described above. A mean association time across the two phases for each size of female was then calculated. Males that did not investigate both preference zones within the initial 10 minute period were excluded and these males were re-tested once more, with new females, 2-11 days after their initial trial. In total, 5 males were re-tested. Of the 17 original males, 15 individuals completed the experiment.

5.3.2 Visual cues test

Here, each male was allowed to choose between a large and small female based purely on visual cues in the complete absence of chemical cues. The experimental set up consisted of three tanks (see Figure 5-1b). The male experimental tank was identical to the tank used in the chemical cues test

described previously, with the same size and positioning of preference zones. On either side of the experimental tank were two 8 litre female tanks (32 x 17 cm x 19 cm high). Between the female tanks and the experimental tank, I attached a piece of one-way film fixed to transparent Perspex. In order for the one-way film to work, a light gradient was created by switching off the main overhead lights and placing a lamp at either end of the experimental set up, illuminating each of the female's tanks. This prevented the females from viewing the male, but allowed the male to see both females. In this way I controlled for any effects that female behaviour may have had on the male's choice. The two lamps provided enough light for the human observer to see the experimental male in the mirror overhead.

A large and small female were chosen and randomly placed on either side of the experimental tank. Large females (mean ± S.E. = 42.94 mm ± 0.83, range = 38.2 – 49.2 mm, N = 14) had significantly greater SL than small females (mean ± S.E. = 29.51 mm ± 1.21, range = 17.1 – 35 mm, N = 14; t- test; t = 9.13, N = 14, $P < 0.001$). The focal male was then placed in a clear plastic cylinder, (diameter 10 cm) in the middle of the tank for 30 minutes allowing him to acclimate and see, but not to associate, with the stimulus females prior to the experiment. The cylinder was then raised on a pulley and moved out of sight. Care was taken to lift the cylinder when the male was not directly facing either female so as not to bias him to initially swim towards one of the females. The focal male was then observed for 10 mins. The amount of time spent in either preference zone whilst oriented towards the female in that side of the tank was recorded. When the male had turned more than 90° away from the female the timer was stopped. I also noted how many sigmoid bend displays the male performed for each female. Sigmoid displays or s-bends are a characteristic sexual display of many species of swordtails, including *X. hellerii* (Rosenthal et al., 1996) whereby the male arches his body into an S shape along the horizontal axis and quivers whilst being oriented towards the female. To control for potential side bias, the females were swapped over, and the second phase was then run as described above. Again, a mean association time for each size of female was calculated from the two phases of the visual cues test.

Before the next male was tested, at least half of the water was changed in the male experimental tank to dilute any chemical cues left over. In cases where a

male did not behave normally (e.g. hid in a corner or remained motionless for the duration of the experiment), a full water change was done afterwards to remove any alarm chemicals etc (N = 3). Data from males who did not investigate both preference zones within the 10 minute period were excluded. These males were retested once more, with new females, 2-8 days after their first trial (N = 5). Of the 17 original males, 14 individuals completed the experiment.

5.3.3 Data analyses

All data were analysed using SPSS version 15.0. Data were checked for normality and homogeneity of variance and where these assumptions were not met non-parametric tests were used. Paired t-tests were carried out to establish if males spent more time associating with large or small females for both the chemical and visual cues tests. I further tested for a relationship between the difference in size of the female dyad (both female SL and gravid patch area) versus the difference in the amount of time that males spent associating with the chemical or visual cues of large and small females. I carried out bivariate Pearson's and Spearman's rank correlations. All tests were two-tailed and where appropriate mean $\pm$ SE are displayed.

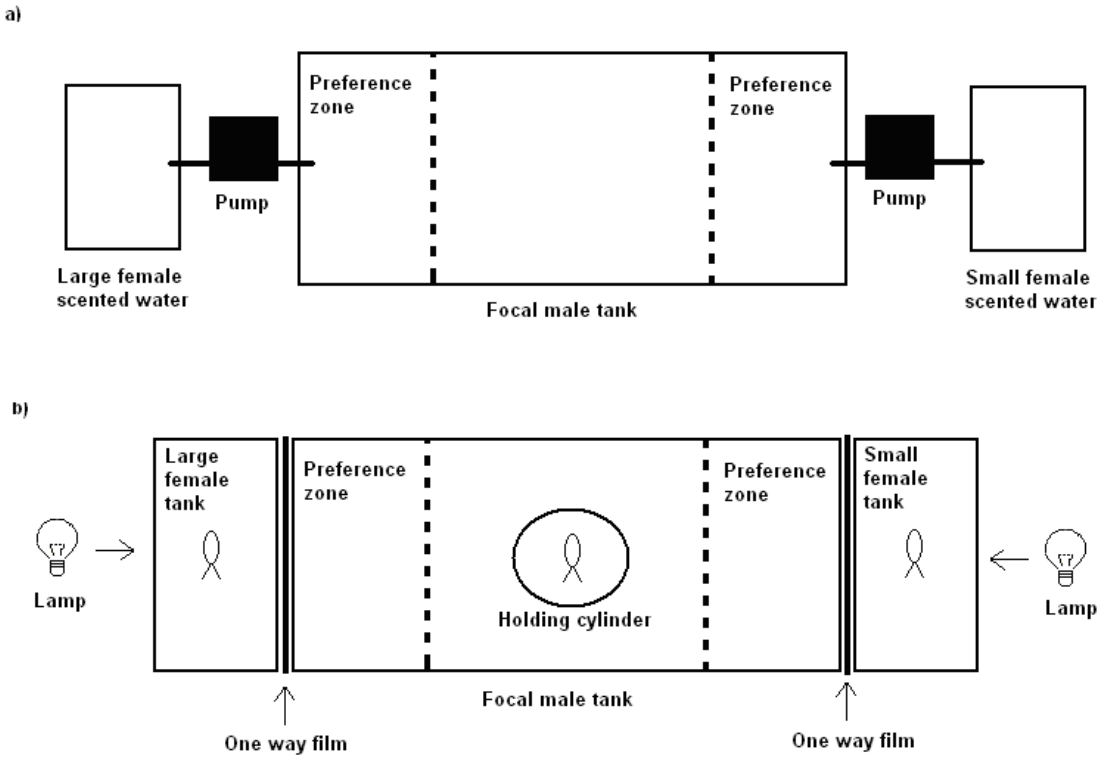

Figure 5-1. Plan of experimental tanks for swordtail male mate choice experiments. Dashed lines indicate 'preference zones' a) Chemical cues test: Solid lines from the pumps to the focal male tank and the female scented water tanks represent the silicone tubing. b) Visual cues test: The circle in the middle of the 'no preference zone' represents the cylinder in which the male was initially held to acclimate. Thick dark lines between the female and male tank on either side are the one-way film allowing the male to view the females but not vice versa. Lamps at either end of the experimental tank created a light gradient. Diagrams are not to scale.

5.4 Results

5.4.1 Chemical cues test

Males swordtails did not spent significantly longer with the chemical cues of either the large or the small female (Paired t-test, t_{14} = 0.27, P = 0.79; Figure 5-2a). There was a significant relationship between the difference in the SL of the female dyad (large female SL – small female SL) and the relative preference of males for large females (time spent with large female – time spent with small female) (Spearman's rho; r_s = 0.56, N = 15, P = 0.031; Figure 5-2b). So, as the size difference between the two females increased, males spent relatively longer associating with the chemical cues of the larger female. There was no correlation between male SL and the difference in association time (Spearman's rho; r_s = -0.23, N = 15, P = 0.46). So, male size did not influence the size of female with which males associated.

5.4.2 Visual cues test

Male swordtails spent significantly longer with large over small females when presented with only visual cues (paired t-test, t_{13} = 2.18, P = 0.049; Figure 5-3a). There was a non-significant trend for males to spend relatively longer with larger rather than smaller females as the difference in the size of the female dyad increased (Pearson's correlation; r = 0.46, N = 14, P = 0.09; Figure 5-3b). Male SL had no effect on the relative amount of time the male spent with the large female (Pearson's correlation; r = -0.23, N = 14, P = 0.43). Large females (mean ± S.E. = 12.58 mm^2 ± 1.44) had larger gravid patches than small females (mean ± S.E. = 3.06 mm^2 ± 0.58; Mann Whitney U test; U = 0.0, N = 28, P < 0.01). However, there was no relationship between the male's relative preference for large females and the difference in gravid patch area between large and small females (Spearman's rho; r_s = -0.257, N = 14, P = 0.38). The number of sigmoid displays performed by the male did not differ between the large and the small females (Wilcoxon signed–ranks test; Z = -1.45, N = 14, P = 0.18). One male performed an unusually large number of sigmoid displays, but the results did not change when this outlier was excluded.

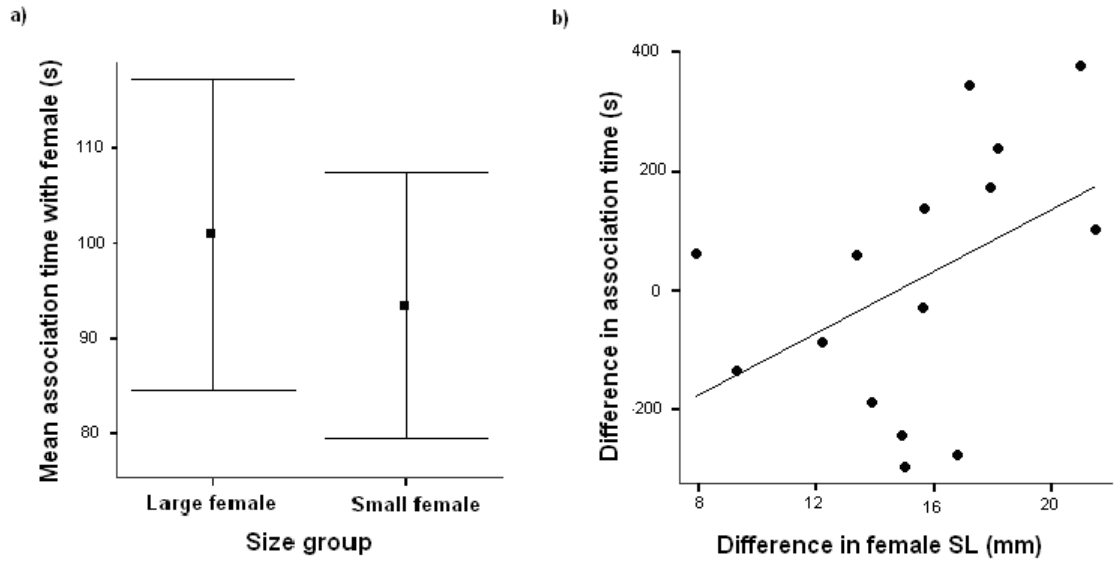

Figure 5-2. Chemical cues test for swordtail male mate choice; a) Mean time (± SE) spent by male with the chemical cues of large and small female. b) Relative male preference for the larger female (total time the male spent with the large female minus the total time spent with the small female) versus the size differential of the female dyad (SL of large female minus the SL of small female).

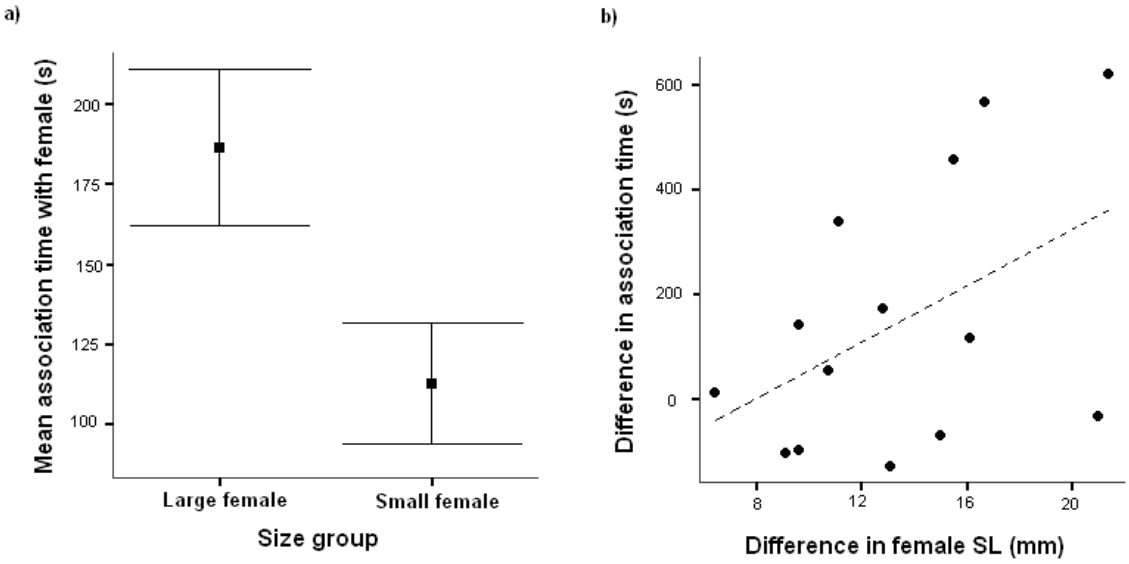

Figure 5-3. Visual cues test for swordtail male mate choice; a) Mean time (± SE) spent by male with the visual cues of large and small female; b) Relative male preference for the larger female (total time the male spent with the large female minus the total time spent with the small female) versus the size differential of the female dyad (SL of large female minus the SL of small female).

5.5 Discussion

My results add to growing evidence in fish that males engage in mate choice for larger bodied females. I found that males significantly preferred large over small females when they were exposed only to their visual cues, but showed no overall preference when exposed to only their chemical cues. Interestingly, as the difference in the SL of the female dyad increased, males spent relatively more time with the chemical cues of the larger females and marginally longer with the visual cues of the larger female compared with those of small females. Thus, males showed a preference, based on visual cues, for a larger over a smaller female even when the size difference between them was small. However, chemical cues only stimulated a male to show a preference for larger over smaller females when there was a sufficiently large difference in female size, and presumably therefore in the amount of chemical stimulus they produced. So overall, visual cues stimulate a stronger mate choice preference in *X. hellerii,* based on female body size, than chemical cues. Contrary to my predictions, males were not found to court large females relatively more than small females, and there was no correlation between the relative size of the gravid patch of the female and relative male preference.

My findings show that male swordtails made mate choice preferences for larger over smaller females based on visual cues. Swordtails often live in clear shallow waters, so visual cues of mates, such as their body size, should be quick and easy to quantify for suitors. Female size has been shown to reflect fecundity, with large females being more fecund than small females (Bagenal & Braum, 1968; Kraak & Bakker, 1998). Work on *X. hellerii* females has found that larger females give birth to heavier fry (Walling, 2006), and heavier fry or fry from larger eggs in other species show increased survival (Einum & Fleming, 1999; Marsh, 1986). Thus, by showing preferences for larger females, male green swordtails may enhance their fitness (See Appendix I, where I investigated the fitness benefits of breeding with large over small females). Alternatively or perhaps additionally, males may attain fitness benefits because larger females may simply be easier for them to detect. By locating and assessing females more quickly, males may reduce their time and energy costs (Pomiankowski, 1987), not to mention predation risk (Johnson & Basolo, 2003; Magnhagen, 1991). For

example, male three-spine sticklebacks, *Gasterosteus aculeatus*, decrease their courtship displays towards females in the presence of a predator (Candolin, 1997).

I found a weaker preference based on chemical cues compared to visual cues in mate choice in male *X. hellerii*. Males did not show a distinct preference for large over small females based only on their chemical cues, but as the size differential of female dyads increased, males spent relatively more time with the larger female. This indicated that larger females produce a greater quantity of their chemical signature compared to small females or a different chemical signal altogether. I used a conservative flow rate of 1.4ml/min, as it has been shown to evoke a response in the swordtail species *X. cortezi,* for females to show preferences for conspecific males (McLennan & Ryan, 1997). However, it is possible that this flow rate did not produce a strong enough stimulus to induce males to show a distinct preference for the large over the small females. Chemical cues may become more important when other cues are obscured, perhaps in turbid streams after heavy rains or in separated murky pools in the dry season. As the fish in my set up had clear still water it is possible that they were not stimulated to respond to the chemical cues of the females. Further work looking at reactions to chemical cues in murky water would resolve this. Although olfactory cues have been found to be important in communication in the aquatic environment, being used for recognition of species (Wong et al., 2005), individuals (Brown & Smith, 1994), MHC-similarity of potential mates (Reusch et al., 2001b) and kin (Brown & Brown, 1996; Le Vin et al., 2010a), little is actually known about the chemical composition of the compounds used by fish and how they differ between individuals. It would be interesting in the future to investigate the compounds that female *X. hellerii* are producing and whether they differ in quantity and/or composition between individuals.

Contrary to my predictions, I found no difference between the number of sigmoid courtship displays performed by the male to either the large or the small female. It is possible that I found no relationship because the females were blind to the male's courtship due to the one way film. In the wild, male *X. hellerii* would be exposed to a full range of sensory stimuli from the females, and females would also be able to respond to the male courtship. Hence, in my set up males may just have given up courting an unresponsive female. Previous work

by Benson (2007) found that male *X. hellerii* courted females with artificially enlarged gravid patches more than control females, although prior to the manipulation females were equally attractive. Even though I found that large females had relatively larger gravid patches than small females, contrary to my predictions I found no correlation between gravid patch area and male association time or courtship rate. However, the colour of the patch, which was also assessed in Benson's study, was not taken into account, so it is possible that attributes other than size are considered by females.

In conclusion, my study adds to the growing evidence for male mate choice. *X. hellerii* is a promiscuous species in which males provide only sperm and show no paternal care so would seem an unlikely candidate for male mate choice. On the other hand, as male swordtails in the wild have access to a range of females who probably differ in quality, there are predicted fitness advantages of being choosy. Males may select a larger, more fecund, female to increase their reproductive output (see Appendix I), or they may receive direct fitness benefits by simply choosing a more detectable female. Alternatively, it is possible that there may not be any benefits to males of being choosy. Preferences by males for relatively large females may just be a by-product of correlated selection of female preference for large male body size. Therefore, it would be interesting for future work to investigate what, if any, fitness benefits male swordtails receive through being choosy.

Chapter 6: General discussion

6.1 Summary of thesis

In my thesis I principally investigated the kin recognition abilities of the cooperatively breeding cichlid, *Neolamprologus pulcher*, and subsequently, whether being able to recognise kin affected helping effort and/or inbreeding avoidance. In chapter 2, I found that *N. pulcher* can recognise unfamiliar kin and that chemical cues played a more important role in kin recognition than visual cues. As *N. pulcher* can recognise kin they may preferentially choose kin with which to cooperate, in order to gain kin selected benefits. In chapter 3, I investigated this and tried to disentangle if helping was driven by kin selected or direct fitness benefits. Further, I investigated if factors other than relatedness may influence helping. My study showed that relatedness did not influence helping effort. However, whilst controlling for relatedness, I found that differences in behavioural type influenced the helping effort shown by *N. pulcher* helpers, with more aggressive, active or risk-prone individuals carrying out more territory defence than less aggressive, inactive and risk-averse individuals. Having found that *N. pulcher* could discriminate between kin and non-kin, but didn't use their kin recognition abilities to preferentially help relatives, it may be expected that they would avoid kin as mates when sexually mature, to avoid the deleterious consequences of inbreeding. However, evidence is building that inbreeding may not always be detrimental to an individual's fitness (Peer & Taborsky, 2005; Sagvik et al., 2005; Waser & Price, 1989). In accordance with this, in chapter 4, I found that *N. pulcher* did not actively avoid kin as mates, and that, in fact, there was no evidence of inbreeding being detrimental to hatching success. A final theme of my thesis was mate preferences based on phenotypic traits. In chapter 4 I found that the size of the facial stripes in *N. pulcher*, had no influence on preferences for associating with an opposite sex conspecific. I also investigated male mate choice for female body size in a non-cooperatively breeding fish species, the green swordtail, *X. hellerii*. Here, I found that males were choosy and preferred larger over smaller females based on visual cues, with chemical cues playing a lesser part in this preference.

In this chapter, I will further discuss these results, including the limitations of them, and how they add to the current literature. Further, I will consider some possible avenues of further research that have been highlighted by my results.

6.2 Kin recognition in *N. pulcher*

6.2.1 Method of kin recognition

Studies investigating kin recognition within a species need to account for familiarity between test individuals, as familiarity does not necessarily constitute relatedness. Familiarity between individuals can form very quickly; for example, in guppies, *Poecilia reticulata*, females become familiar with one another after only 12 days (Griffiths & Magurran, 1997), and in sticklebacks, *G. aculeatus*, context dependent preferences for individuals can form after only 24 hours (Ward et al., 2005). Thus, it is important to control for familiarity from birth in kin recognition experiments. My study in chapter 2 used a carefully controlled breeding design, which separated eggs before they hatched, and found that *N. pulcher* could discriminated between unfamiliar kin and unfamiliar non-kin, using either self-referent or non-self referent phenotype matching. As *N. pulcher* broods in the wild can contain eggs sired by different males (Dierkes et al., 1999; 2008; Heg et al., 2006), being able to recognise kin via self-referent phenotype matching would be the most reliable way to identify true relatedness to other conspecifics. Disentangling whether *N. pulcher* use self-, or non-self referent phenotype matching would be a possible avenue for future research. However, in *N. pulcher* teasing apart these two recognition methods would be problematic. One way would be to cross-foster a single individual before hatching into another brood, and then later testing that individual to see if they could recognise kin; hence, showing self-referent phenotype matching. Alternatively, if they recognised unfamiliar conspecifics related to their foster brood as kin, they must use non-self referent phenotype matching (for review see Mateo & Holmes, 2004). Mateo and Johnston (2000), cross fostered single golden hamsters, *Mesocricetus auratus*, into new broods and found they could recognise unfamiliar kin via self-referent phenotype matching. However, to carry this out, individuals within broods need to be easily distinguishable, or marked to maintain their identity, and currently this would be almost impossible

in *N. pulcher* due to the fry being so small. Alternatively, a single individual could be raised either in total isolation, or removed from its foster brood but still be in chemical and visual contact with them, before testing its kin recognition capabilities. However, raising such a social species in isolation may influence an individual's behaviour and would present an ethical conundrum. Hence, in *N. pulcher*, teasing self- from non-self referent phenotype matching may be impractical and unethical.

6.2.2 Cues used for kin recognition

In chapter 2, I found that chemical, rather than visual cues were more important for kin recognition in juvenile *N. pulcher*, as has been found in other species (Mehlis et al., 2008; Neff & Sherman, 2005; Olsen et al., 1998). Chemical cues are more likely to be picked up at a greater distance than visual cues, which may be obscured in murky water in aquatic species. However, it is currently unknown exactly what chemical cues *N. pulcher* use to recognise kin. Genes of the major histocompatibility complex (MHC) are highly polymorphic and encode glycoproteins that are involved in the recognition of self and non-self antigens in the immune system in vertebrates (Penn & Potts, 1999). Furthermore, they have been found to influence odour, though exactly how they do this, is as yet unclear (Penn & Potts, 1999). MHC genes have been implicated in the control of chemically-mediated kin recognition and mate choice in several species (e.g. Arctic charr, Olsen et al., 1998; mice, Yamazaki et al., 1976; sticklebacks, Reusch et al., 2001a; and humans, Wedekind & Furi, 1997). Individuals have been shown to recognise kin sharing similar MHC alleles, or select MHC dissimilar or diverse mates to avoid inbreeding and/or to produce genetically diverse offspring. Therefore, it would be interesting to investigate if *N. pulcher* use MHC similarity to assess kinship. However, there has been recent debate surrounding the MHC and its role in recognising relatedness between potential mates, at least in mammals (Hurst, 2009). Most of the work investigating the MHC is based on inbred strains of laboratory mice, and results have varied depending on the strain and sex tested (Jordan & Bruford, 1998). More recent work has focused on other polymorphic genetic markers that may be used for recognition. Major urinary proteins (MUP's) are small lipocalin proteins that can bind lipophilic molecules such as volatile male pheromones (Robertson et al., 1993), so are

implicated in chemical signals. MUP's are highly polymorphic, so only close relatives are likely to share the same haplotypes. Work on freely breeding wild mice has found that mates with the same MHC are not avoided, whereas mates sharing both MUP haplotypes are (Sherborne et al., 2007). So far, it is unknown whether fish produce MUP's and consequently, it would be interesting in future studies to investigate whether they are being used for chemical kin recognition and/or avoidance of breeding with genetically similar individuals.

6.3 Helping in *N. pulcher*

6.3.1 Effect of relatedness on helping

Developing the mechanisms with which to recognise kin is likely to be costly, so there must be a reason as to why *N. pulcher* can recognise kin. It was thought for a long time in cooperatively breeding groups that helpers were the past offspring of the breeders, and aided their parents to gain kin selected, indirect, fitness benefits (Hamilton, 1964b). However, this is now known not to be the general rule for cooperatively breeding species, with groups containing related as well as unrelated helpers (for example; Dierkes et al., 2005; Magrath & Whittingham, 1997; Van Horn et al., 2004). Further, theoretical work suggests that competition arising between relatives in cooperative groups can actually cancel out any indirect fitness benefits they may gain (for review see Griffin & West, 2002). Instead, helpers, both related and unrelated, may aid breeders for the direct fitness benefits they gain through group living (Clutton-Brock, 2002; Griffin & West, 2002). However, trying to disentangle the effects of relatedness on helping effort in cooperative groups is often difficult, particularly as genetic relatedness between individuals needs to be known, either from pedigree data, which is often not 100% accurate and takes a long time to collect (Emlen & Wrege, 1988; Komdeur, 1994), or from genetic analysis (Clutton-Brock et al., 2000). As described in chapter 3, prior work investigating whether relatedness of helpers to breeders influenced helping effort, and thus whether helpers aided more for indirect or direct fitness benefits, found inconsistent results in *N. pulcher*. Stiver et al (2005), found in the laboratory, that unrelated helpers helped most with both digging and defence of the territory. In the field, on the other hand, helpers related to the breeding female and helpers unrelated to the

breeding male, carried out the most defence, but not more digging on the territory (Stiver et al., 2005). In my opinion, this inconsistency between captive and wild behaviour was due to the fact that there were no manipulations carried out to standardise the amount of helping effort required from a group. Instead, the study just looked at natural levels of helping, which is likely to have varied considerable between groups. Thus, Stiver et al's (2005) study could not distinguish with any certainty whether indirect or direct fitness benefits were more important in influencing *N. pulcher* to help. I used a carefully designed experiment in chapter 3, controlling for relatedness, size and familiarity within groups. Further, I carried out standardized helping experiments, so I could be sure that each helper had equal opportunity to display helping behaviour. From these experiments, I was able to ascertain that the relatedness of the helpers to the breeders did not influence helping effort. Therefore, in *N. pulcher*, kin selected benefits alone cannot explain variation in helping behaviour. Related helpers will still receive kin selected benefits, in addition to direct fitness benefits. But how great these kin selected benefits are is unknown. Unrelated helpers, on the other hand, will receive only direct fitness benefits. Thus, these must be either equal to the benefits the related helpers receive to compel them to help as much, or, unrelated helpers may have to 'pay to stay' more than a related helper to be tolerated by the breeders, as has been proposed by Kokko et al (2002). In order to ascertain how important kin selected benefits are, experiments need to be carried out assessing the helping effort of an individual living in a group of only related conspecifics, versus them living in a group of mixed relatedness, and a group of only unrelated individuals. If kin selected benefits are important, I would expect them to help most for groups of only relatives, help least for groups of only non-relatives, and show intermediate levels of helping for mixed relatedness groups. However, if the direct fitness benefits gained through group living are more important, then they should help equally in all situations. The importance of direct fitness benefits may be being underestimated (Clutton-Brock, 2002; Griffin & West, 2002), and in some cases could actually be driving all individuals to help, regardless of relatedness. In the spotted hyena, *Crocuta crocuta,* relatedness within groups is low; hence, individuals must cooperate for the direct fitness benefits gained through group living, such as gaining and retaining access to food resources (Van Horn et al., 2004). So, direct fitness benefits can be more than sufficient to stimulate

individuals to help. Additionally, in meerkats, *Suricata suricatta*, the amount of helping by subordinates in terms of babysitting and feeding pups has been found to be driven by the age, sex and weight of helpers, rather than relatedness to the pups (Clutton-Brock et al., 2000; 2001). Hence, variation between individuals may also be important in influencing how much help is shown.

One problem I faced in my study in chapter 3, was trying to set up social groups of *N. pulcher*, as often helpers were not accepted straight away into the group. In natural groups of *N. pulcher*, there is a size dominance hierarchy, with larger individuals being dominant over smaller ones (Taborsky, 1984; Taborsky & Limberger, 1981). In my study, helpers were size-matched, to control for any influence size had on an individuals willingness or ability to help. It is possible that a lack of size difference between the helpers influenced group cohesion. Thus, my study could perhaps have been improved by using helpers that varied in size as well as relatedness, and controlling for helper body size in the analysis.

6.3.2 Effect of behavioural type on helping

My work in chapter 3 highlighted that there were individual differences in the amount of helping effort carried out by *N. pulcher* helpers, which could not be explained by relatedness. The animal personality literature has been touched upon in chapter 3. This subject area is relatively new and fraught with discussion over terminology and definitions (for reviews see Realé et al., 2007; Sih et al., 2004b), which is beyond the remit of this thesis. However, evidence is being rapidly gathered showing that many species show consistent-within and variation-between individuals in different behaviours, or exhibit 'behavioural types' (Alvarez & Bell, 2007; Bell, 2005; Bell & Sih, 2007; Bell & Stamps, 2004; Cote & Clobert, 2007; Dingemanse et al., 2002; 2003; 2007; Drent et al., 2003; Herborn et al., 2010). Studies have also investigated how these differences in behavioural types then affect behaviours that influence fitness, such as foraging (Herborn et al., 2010), dispersal (Dingemanse et al., 2003), and in *N. pulcher*, helping (Bergmüller & Taborsky, 2007; Schürch & Heg, 2010). As discussed in chapter 3, I investigated the effects of differences in behavioural types on helping effort, but importantly, unlike previous studies, I also controlled for the

potential effects of relatedness. When I compared my results to those of Schürch and Heg (2010) and Bergmüller and Taborsky (2007), I found inconsistencies between our findings, with each study identifying different behavioural types and syndromes. Hence, my work adds to the evidence building that behavioural types and their associated behavioural syndromes can differ between populations of the same species (Alvarez & Bell, 2007; Bell, 2005; Bell & Sih, 2007; Bell & Stamps, 2004; Dingemanse et al., 2007). Different populations can have different pressures acting upon them, which may shape the behaviours they show. For example, Alvarez and Bell (2007) found that stickleback populations from streams were bolder than stickleback populations from ponds. Further investigation found that differences in behaviours between populations was linked to the presence or absence of predators (Dingemanse et al., 2007). However, why differences were found in behavioural types between my population of *N. pulcher* and those in other studies is currently unknown, as all fish were raised under similar conditions in captivity. This therefore, warrants further investigation. Differences in behavioural types may arise from individuals having been collected from different areas of Lake Tanganyika, where selections pressures may vary, from differences in how many generations individuals have been bred in captivity, or perhaps from social experiences, which I will discuss further below.

Whilst my study has found that individuals with certain behavioural types helped more, it could not explain why this was the case. Furthermore, I found that individuals that carried out more territory defence also carried out more maintenance, so some individuals are generally more helpful than others. For helping to be worthwhile, the benefits received must be greater than the costs paid (Emlen, 1982). So these helpful individuals must either be receiving greater benefits, or paying fewer costs, than less helpful individuals. It is possible that more aggressive, active or risk-prone individuals may be in better condition or fitter than less aggressive, inactive or risk-averse individuals. They may for example, be better at obtaining food. Thus, they may be able to afford to help more and/or pay less cost in their helping effort. However, in chapter 3, as I size matched my pairs of helpers, and all fish had been kept in the same conditions under the same feeding regime, it seems unlikely that they would have differed substantially in condition. Aggressive, active or risk-prone individuals were found

to carry out more territory defence, a helping behaviour that carries a risk of injury through fighting (Balshine et al., 2001), consequently, they would be expected to receive substantial benefits. Aggressive and more risk-prone individuals in some species have been found to be more likely to be dominant in a group (Robert et al., 1988; Sundström et al., 2004; Verbeek et al., 1996). It is possible that aggressive or more risk-prone individuals that help more, may move up the dominance ranking of the group quicker than individuals that are less aggressive and help less, although this hypothesis needs to be tested. By moving up the dominance ranking, individuals may gain benefits such as securing a breeding position (Arcese & Smith, 1985), increasing their reproductive success (Paull et al., 2010) or it may allow them access to the breeders to sneak matings. However, the reason why individuals with certain behavioural types help more in *N. pulcher* remains unclear and untested, and thus, further investigation is required to understand exactly what benefits these individuals gain by doing so.

6.4 Effects of early social environment

Recent work on *N. pulcher* has found that an individual's early social environment can have subsequent effects on its social behaviours later in life (Arnold & Taborsky, 2010). For these experiments, individuals were either raised from fry with breeders and/or older helpers present (so called F+ helpers), or raised without breeders and/or older helpers (so called F- helpers). F+ helpers showed more aggressive and submissive acts towards each other than F- helpers. However, when given a social role as either a shelter owner, or intruder to another individual's shelter, F+ and F- helpers differed in their behaviours. F+ helpers showed more restrained, and less energetically costly aggressive behaviours when they were the shelter owner, and more submissive behaviour when they were a shelter intruder compared to the F- helpers (Arnold & Taborsky, 2010). Thus, helpers raised with family appear to be better at adjusting their behaviour to suit the social situation than helpers raised without adults. This may help to resolve conflicts more quickly and reduce the chance of injury or expulsion from the territory. The fish used in my experiments were all raised without adult supervision. This was essential in order to control for familiarity between individuals. As a result, however, this could have had effects

on the behaviours of the individuals. In contrast, other studies in *N. pulcher* investigating the effects of relatedness and behavioural types on helping effort have used individuals that had been raised with older group members (Bergmüller & Taborsky, 2007; Schürch & Heg, 2010; Stiver et al., 2005). This may in part explain some of the differences found between results. In spite of this, it is important to point out that all of the individuals used in my study still showed the normal behaviours seen in *N. pulcher* (Taborsky, 1984), such as aggression and submission towards conspecifics, defence against intruders, digging sand from the territory and defending against intruders. Further, as all individuals experienced the same social rearing environment, this should not have systematically biased any of my results. However, being reared without adults, may in part explain the problems I experienced setting up social groups of *N. pulcher* in chapter 3. It seems likely that helpers may not have initially behaved in an appropriate manner to the breeders, and hence may have taken a longer time to be accepted into the territory, than if they had been reared with adults. If the early rearing environment can affect social behaviours in *N. pulcher*, then it is possible that it could also have effects on other behaviours, such as helping effort or individual behavioural types, although this has yet to be explicitly tested.

6.5 Kin recognition and inbreeding in *N. pulcher*

In chapter 2, I found that juvenile *N. pulcher* can recognise and show discrimination between kin and non-kin. However, in chapter 3 I found that the ability to recognise kin did not influence helpers to aid relatives more than non-relatives. Therefore, it would seem reasonable to think that as adults, *N. pulcher* may use kin discrimination to avoid inbreeding. However, a field study found that adults breed at random with respect to relatedness (Stiver et al., 2008) and so do not actively avoid inbreeding. Nevertheless, Stiver et al's (2008) study found that allele sharing scores (i.e. relatedness scores) between the breeders were generally low, with a mean relatedness of $r = 0.011$. Further, they were unable to confirm if alleles that were shared were identical by descent, and hence from true relatives, or just shared by chance. In addition, they did not investigate any differences in breeding success between related and unrelated pairings. Field studies are essential to assess how individuals breed

under natural conditions, but breeding behaviour in the wild may be mediated by constraints placed on an individual, such as a lack of potential breeding partners, or breeding territories. For example, naked mole rats, *Heterocephalus glaber,* showed high levels of inbreeding in the wild (Reeve et al., 1990) presumably as there was little dispersal between colonies. However, when given the option to breed with familiar close kin or unfamiliar distant kin in captivity, they preferred distant kin (Ciszek, 2000). So, captive studies can help to remove social and environmental factors that may drive an individual to make a suboptimal mate choice. My study in chapter 4 investigated the propensity to breed with first order relatives (r = 0.5), or completely unrelated conspecifics (r = 0). The results of my study reinforced the findings of Stiver et al (2008), as *N. pulcher* were found to breed as readily with siblings as with non-siblings. Therefore, even first order relatives are not avoided as mates, when the alternative is to not breed at all. Furthermore, I also assessed the fitness consequences of inbreeding, and found no disadvantage to breeding with kin over non-kin, and even that breeding with relatives may increase hatching success. This may partly explain why inbreeding is not avoided in this species. As covered in chapter 4, it seems likely that inbreeding in *N. pulcher* is not the norm, but instead is a trade-off against not breeding at all. My study, nevertheless, could have been improved and expanded upon by investigating the longer-term effects of inbreeding on *N. pulcher*. For instance, investigating survival of offspring to reproduction and the lifetime fecundity of offspring, as the effects of inbreeding may not immediately be apparent. Furthermore, it would be interesting to investigate mating preferences when *N. pulcher* are presented simultaneously with a related and unrelated partner, as it has been proposed that inbreeding may be less likely when individuals have simultaneous rather than sequential mate choice (Kokko & Ots, 2006). This would help to disentangle whether inbreeding is an active choice, or is due to limitations on breeding opportunities.

Laboratory studies of inbreeding, as well as kin recognition, often have a major flaw. These studies need to create groups of ‘related’ and ‘unrelated’ individuals with which to test inbreeding avoidance or recognition of kin. Generally, the breeders used to produce the offspring for these groups are assumed to be unrelated (Arnold, 2000; Gerlach & Lysiak, 2006; Griffiths &

Magurran, 1999; Olsen, 1989), but this is not verified. Consequently, there is a possibility that the population used are already showing signs of inbreeding, such as an excess of homozygotes. This is particularly likely when stocks have been bred in captivity over multiple generations. This could potentially confound results, as the 'related' and 'unrelated' groups produced may be genetically very similar, making them hard to distinguish. My study is novel in that it used molecular techniques to establish that the original stock of breeders used did not show any indication of prior inbreeding. Thus, I could be certain that any avoidance, or preference of kin over non-kin as mates, was not confounded by individuals being more genetically similar than expected. Future studies really need to explore the amount of allele sharing between breeders used for inbreeding experiments, in order to make certain that their results are not confounded by already inbred individuals.

6.6 So why do *N. pulcher* show kin recognition?

In my thesis, I have found that *N. pulcher* can recognise kin. However, kin recognition does not influence *N. pulcher* helpers to aid relatives more, or prevent them from inbreeding. Thus, the question remains as to why *N. pulcher* can recognise kin? It is possible that there may be alternative benefits gained through association with kin. In rainbow trout, *Oncorhynchus mykiss,* individuals in kin groups showed lower levels of aggression and increased weight gain over groups of non-kin (Brown & Brown, 1993a; 1993b) and aggression was reduced in kin groups of brown trout, *Salmo trutta* (Olsen et al., 1996). An increase in weight may also increase body condition, which could have effects on survival, as has been found in other species (Hoey & McCormick, 2004; Naef-Daenzer et al., 2001; Thompson et al., 1991). Consequently, it would be interesting to carry out further experiments to assess whether living with kin has fitness advantages, such as reduced aggression or weight gain, over living with non-kin, or in groups of mixed relatedness. In my helping experiments in chapter 3, I used small social groups of *N. pulcher,* containing only two helpers, one related and one unrelated to the breeders. However, groups in the wild can have up to 14 helpers of mixed relatedness (Taborsky & Limberger, 1981). My simplified social groups may have required that both helpers had to help equally in order to satisfy the helping requirements of the breeders. Therefore, it is possible that in more complex

groups with more helpers, individuals may behave differently and perhaps show selection to help kin over non-kin. Alternatively, individuals may be more likely to show kin selection when under threat, for example if a predator was present. This may encourage them to help to protect relatives with whom they will share genes, over non-relatives. As discussed previously, *N. pulcher* may also show more discrimination in their mate choices if they are given simultaneous rather than sequential mate choices. It is proposed that individuals may be more willing to inbreed when only presented with one mate, and hence, one opportunity to breed at a time (Kokko & Ots, 2006). In conclusion, further research is required to try and tease apart the importance of kin selected over direct fitness benefits for helpers in *N. pulcher*. In addition, more work is required to ascertain if inbreeding is an opportunistic behaviour, or if it is a strategic choice. With further studies, hopefully light can be shed on why *N. pulcher* can recognise kin.

6.7 The influence of facial stripes on preferences for opposite sex conspecifics in *N. pulcher*

Having found that kin recognition in *N. pulcher* was not used in mate selection, I investigated whether the two facial stripes present on the operculum had any influence on preferences for conspecifics of the opposite sex. Until now, other studies on *N. pulcher* have not directly investigated any potential functions of the facial stripes. I found no evidence to suggest that facial stripes were used by *N. pulcher* to show preferences for individuals, either kin or non-kin. However, I think that facial stripes still warrant further investigation. From my own personal observations I have noted that facial stripes develop first in the largest individual in a group. Thus, it seems plausible that facial stripes may develop as an indicator of dominance or a 'badge of status' (Rohwer, 1975). Dominant individuals are often chosen as mates; for example, in collared flycatchers, *Ficedula albicollis,* males with larger white forehead patches are dominant over other males and gain territories and mates quicker (Pärt & Qvarnström, 1997). I think it would be interesting to examine whether individuals that developed their facial stripes first in a group are also dominant over other group members, and further, if they are more likely to obtain a breeding position than group members that developed their facial stripes later. In other cichlids, colouration has also been found to be used by females to distinguish between males of

different species (Seehausen & Alphen, 1998). So, it is possible that aspects other than the size of the facial stripes may influence mate preferences, or recognition of conspecifics, such as the intensity of the colour of the stripes, or the colours surrounding the stripes. The effect of size and colour could be further examined by manipulating facial stripes, and assessing mate preferences. This could be carried out by either dye marking individuals (for example see Benson, 2007), or by using video playback showing fish with manipulated facial stripes (see Balshine-Earn & Lotem, 1998 for evidence of *N. pulcher* responding to video playback of conspecifics).

6.8 Male mate choice in green swordtails, *X. hellerii*

In addition to examining mate preferences based on a phenotypic trait in *N. pulcher*, I also examined male mate choice for female body size in a non-cooperatively breeding, live bearing poeciliid fish, the green swordtail *Xiphophorus hellerii*. Male mate choice is often overlooked because females are expected to be the choosier sex; however, as reported in chapter 5, I found that male *X. hellerii*, preferred to associate with the visual cues of larger over smaller females. Further, I found that chemical cues played a lesser role in male mate choice, with males only stimulated to show a preference when the size difference between the two females presented to them was large. In a species in which males show no parental care and only provide females with sperm, it would be expected that males would maximise their fitness by breeding with as many females as possible (Trivers, 1972). Thus, I would expect that females must differ in their fecundity; otherwise male choosiness would seem counterintuitive. I carried out a breeding experiment in order to assess if larger females were indeed more fecund than smaller females, which would bring the male increased fitness benefits. However, due to females cannibalising fry and low success in breeding I was unable to determine with any certainty if larger females were indeed more fecund. I have outlined in chapter 5 and appendix I, ways in which I believe this study could have been improved. I believe further work is required to establish what, if any, fitness benefits there are in choosing larger over smaller females. Experiments using females of known breeding experience, and using breeding cages that allow fry to be isolated from the female quickly after birth to prevent cannibalism, would give a more accurate

picture of how female size influences fecundity. Further, experiments allowing males chemical, visual and tactile cues of females would be interesting, to assess if males also court larger females preferentially over smaller females, as I found in my experiments that males did not consistently court females that were unresponsive to them. Overall, although I have found male mate choice in green swordtails, the reasons why males are choosy has yet to be established.

6.9 Closing remarks

Kin recognition allows individuals to assess their relatedness to conspecifics. From this they may then show kin discrimination and make informed choices as to with whom to associate and/or breed. Nevertheless, this relies on individuals having developed mechanisms to recognise kin accurately, such as phenotype matching, and ideally self-referent phenotype matching. I found that *N. pulcher* can recognise kin via phenotype matching; however, as shown throughout my study, the ability to recognise kin does not necessarily compel individuals to help kin over non-kin, or even to avoid them as mates. Instead it appears that population-specific differences in individual behavioural types, or constraints on breeding opportunities are more likely to influence an individual's decision to either help or breed. Thus, studies need to carefully consider and explore other fitness benefits that individual may gain through choosing to associate with, or avoid kin. Coupling kin recognition and discrimination with the complexity of behaviours exhibited within cooperatively breeding systems, it is perhaps of little wonder that the reasons behind *N. pulcher* showing kin recognition are unclear. Furthermore, it seems likely that there may be species and even population specific benefits to recognising and aiding or avoiding kin. Hence, only by examining multiple factors affecting fitness, are we likely to understand why different species show a propensity to recognise and discriminate between kin and non-kin.

Appendix I: Does size matter? Do large female green swordtails, *Xiphophorus hellerii*, have increased fecundity over small females?

Abstract

Traditionally, mate choice has focussed on females, but more recently, studies have begun to investigate the importance of male mate choices. Males are predicted to be choosy under certain circumstances, for example, where females differ in quality and/or if mating with one female may reduce the chances of successfully mating with subsequent females. Previously, I found that male green swordtails, *Xiphophorus hellerii*, preferred to associate with larger over smaller females. Here, I investigated if larger female *X. hellerii* are more fecund that smaller females. I used a mixture of virgin females and females of unknown breeding experience in this study. I found no evidence that larger females gave birth to more, better surviving or quicker growing fry, compared to smaller females. Further, large females were not more likely to breed and give birth, compared to small females. However, my results may be confounded by a number of factors, including: cannibalism of fry, small sample sizes and differences in the breeding experience of the females used. I found a non-significant trend for virgin females to have lower fry survival compared to females of unknown experience, but there was no effect of experience on the original number of fry that a female gave birth to. This study highlights the importance of investigating the fitness advantages of being choosy. However, in male green swordtails, the fitness advantages of choosing large over small females remains unclear; thus, further investigation into female fecundity is required.

Introduction

Darwin's (1871) theory of sexual selection predicts that males should mate with as many females as possible to maximise their fitness, whilst females should be choosy and aim only to mate with males of high quality (Trivers, 1972). However, more recently, it has been argued that males too should be choosy

under certain circumstances. For instance, where males have a selection of females that differ in quality, and/or where mating with one female may reduce their chances of fertilizing subsequent females (Andersson, 1994). Mate choice has been extensively studied in females, ranging from studies in birds (Collins et al., 1994; Petrie et al., 1991), to mammals (Clarke & Faulkes, 1999; Drickamer et al., 2000), insects (Borgia, 1981) and amphibians (Gerhardt et al., 1996). In fish, females have also been found to show preferences for male traits, for example, in sticklebacks, females prefer males with more intense red colouration, and these males are fitter than males with dull red colouration (Bakker & Mundwiler, 1994; Milinski & Bakker, 1990). More recently, studies have also been investigating male mate choice (Amundsen & Forsgren, 2001; Byrne & Rice, 2006; Jones et al., 2001). In fish, male mate choice for larger females has been found in several species (Côte & Hunte, 1989; Herdman et al., 2004 and see chapter 5; Kraak & Bakker, 1998; Ptacek & Travis, 1997), as larger bodied females are generally assumed to be more fecund than smaller females (Bagenal & Braum, 1968). However, it is important when investigating mate choice to quantify what, if any, fitness benefits are being gained through being choosy. For example, Herdman et al (2004) found that male guppies, *Poecilia reticulate,* preferred larger females, and larger females gave birth to larger broods.

Male green swordtails, *Xiphophorus hellerii,* have been found to prefer to associate with the visual cues of larger over smaller bodied females (Chapter 5). In the wild, males are likely to come across a range of females differing in size, so being choosy may be advantageous, especially if these females also differ in their fecundity. Larger female swordtails should be more fecund than smaller females, as in fish, fecundity generally increases with female body size (Bagenal & Braum, 1968). In swordtails, one study of wild caught females found that female fecundity was a curvilinear function of female body weight (Milton & Arthington, 1983). However, that study assessed fecundity as the number of embryos found in dissected females, not the number of fry born to females. So, it would be of interest to determine in a controlled experimental set-up, if larger females are indeed more fecund, and give birth to more or better surviving fry than smaller females.

The aim of this study was to ascertain, in the population of *X. hellerii* used for the mate choice experiments in chapter 5, if breeding with larger bodied females leads to males having increased numbers of offspring, over males breeding with smaller bodied females. I also investigated whether larger females are more likely to breed and produce fry than smaller females. Further, I examined whether large females give birth to faster growing or better surviving fry than small females.

Methods

Animal husbandry

All fish used for breeding were third and fourth generation offspring from wild-caught adult *X. hellerii*, from Belize, Central America. Prior to breeding, all fish were maintained in separate sex 50 litre tanks (60 x 30 cm and 30 cm high). Tanks had a weekly 25% water change to maintain water quality and water temperatures were maintained at 24.05 ± 1.05°C, pH at 7-7.4, and light:dark regime at 13.5:10.5 hours. All water was charcoal filtered and aerated for at least 18 hours prior to use in tanks. Fish were fed once daily in the morning, either on commercial flake food or frozen bloodworms.

Breeding experiment

After completion of the behavioural experiments outlined in chapter 5, 'large' and 'small' female *Xiphophorus hellerii* were bred with males to investigate the potential fitness benefits of males being choosy. Two breeding rounds were performed, one in September 2007 and the other in October 2008, using different cohorts of swordtails. For the 2007 breeding round, virgin females were not available. However, all females used did not appear to be pregnant, and had been isolated from males for a minimum of four months, during which time they had not given birth. For the 2008 breeding round, virgin females were used to ensure that they were not pregnant.

Before breeding, measurements of the female's standard length (SL) and mass were recorded and females allocated to either a 'large' or 'small' female

category. Females in the large breeding group had significantly longer standard length (SL) (mean SL = 45.67 mm ± 0.74) and greater masses (mean mass = 2.60 g ± 0.12) than the females in the small group (mean SL = 30.55 mm ± 0.82; mean mass = 0.95 g ± 0.06) (SL; Mann - Whitney *U* test; $U = 0.00$, $N = 24$, $P < 0.001$; mass; Mann – Whitney *U* test; $U = 0.00$, $N = 24$, $P < 0.001$). In each of the two breeding rounds, six 'large' and six 'small' females were selected. Each female was then housed singly in a 32 litre tank (35 x 30 cm and 35 cm high), provisioned with a corner filter. Females were given a week to acclimate to their new tank, before a male was introduced.

Females were given a selection of three males, as previous studies have found that females are more likely to reproduce when mated with a preferred male. Thus, the following protocol increased the likelihood that a female would received a male of their preferred phenotype (Walling, 2006). A selection of 12 mature males were chosen from stock, and randomly assigned to one of the 12 female breeding tanks. Males were then given a period of two weeks with their respective females to breed. After the two weeks had elapsed, each male was randomly assigned to another of the 12 females and given a further two weeks with this new female. Finally, males were, again, randomly assigned to another female's tank and given a final two weeks to breed, before all males were removed completely from the breeding tanks. Care was taken that each of the 12 females were not exposed to the same male more than once.

Tanks were checked daily for fry throughout the breeding period. As fry are sometimes eaten (Jones et al., 2008), each tank was provisioned with a plastic mesh tube weighted down with stones, and a plastic plant, that allowed the fry to shelter away from the adult fish. When fry were found, the female was left in the tank with them for a further day in case she had not finished giving birth. If a male was still present in the tank when the fry were born, he was immediately removed. One day after birth, the female was removed from the tank, and the total number of fry (both alive and dead) was counted. As individual fry were too light to register on a scale to be weighed, fry had to be weighed as a group. From the mass of the group, a mean individual fry mass could be calculated, by dividing the mass of the group by the number of fry. After weighing, fry were released back into their 32 litre tanks and were counted and weighed again on days 7, 14, 21 and 28 in their groups, to assess survival and mass gain.

In the first breeding round, 10 out of the 12 females gave birth, but only five of these females had offspring surviving long enough to be weighed and counted until day 28. In the second breeding round, 10 of the 12 females gave birth and seven of them had fry that survived throughout the experiment. Therefore, a total of 12 females had fry that could be included in the full analysis over the 28 days. Of the other eight females that gave birth, three of them ate their fry and two of them had only dead fry on day 2, so no mass recordings could be collected. The final three females had fry that did not survive past day 2, so only a single mass measurement was recorded. Thus, over the two breeding trials, a total of 20 females gave birth (eleven large and nine small), but my data set for day two (where fry were found alive) has a sample size of 15 (nine large and six small), and when I include both fry found alive and dead on day two I have a sample size of 17 (ten large and seven small). All subsequent data sets have a sample size of 12 (seven large and five small).

Data analysis

All data were analysed using SPSS version 15.0. Data were checked for normality and homogeneity of variance, and where these assumptions were not met non-parametric equivalents were used. A Pearson's correlation was carried out to ascertain if female SL and mass were tightly correlated. All other analyses used only female SL or female size group ('large' or 'small' female). A binomial test was used to assess if large or small females were more likely to give birth. Linear regressions were used to examine the influence of female size on the number of fry produced and fry survival. For all mass analyses on fry I used the mean mass of an individual fry within a brood I also used repeated-measures general linear models (GLM), including female SL as a covariate, and separately, female size ('large' or 'small') as a factor, to test if female size had effects on the mass increase of the fry over the 28 days. Finally, I ran a Mann-Whitney U test and a t-test, to ascertain if the virgin females had lower numbers of fry alive on day 2, or lower percentage fry survival on day 28, than the females whose breeding experience was unknown.

Results

Female SL and mass were found to be highly correlated (Pearson's correlation; $r = 0.97$, $N = 24$, $P < 0.001$). So, female SL was used for analysis on female fecundity. Females from the large group were not more likely to give birth than females from the small group (Binomial two-tailed test, $N = 20$, $P = 0.824$). Female SL did not predict the number of fry found alive on day two (linear regression; $F_{1, 14} = 0.01$, $P = 0.91$; Figure 7-1a), nor did it predict the total number of fry (both alive and dead) found on day two (linear regression; $F_{1, 16} = 0.76$, $P = 0.40$; Figure 7-1b). The percentage survival of fry until day 28 was not related to female SL (linear regression; $F_{1, 11} = 0.05$, $P = 0.82$; Figure 7-2). There was no relationship between the SL of the female and the change in the mean mass of a fry in a brood over the 28 days (repeated measures GLM; $F_{1, 10} = 0.48$, $P = 0.51$). Further, fry from 'large' females did not increase in mass quicker than fry from 'small' females (repeated measures GLM; $F_{1, 10} = 0.78$, $P = 0.40$).

There was no difference in the number of fry found alive on day two for the virgin females, or the females whose breeding experience was unknown (Mann-Whitney U test; $U = 41.0$, $N = 20$, $P = 0.51$). However, there was a non-significant trend for virgin females to have reduced percentage survival of fry on day 28, compared to the females of unknown breeding experience (t-test; $t = 2.01$, $N = 12$, $P = 0.07$; Figure 7-3).

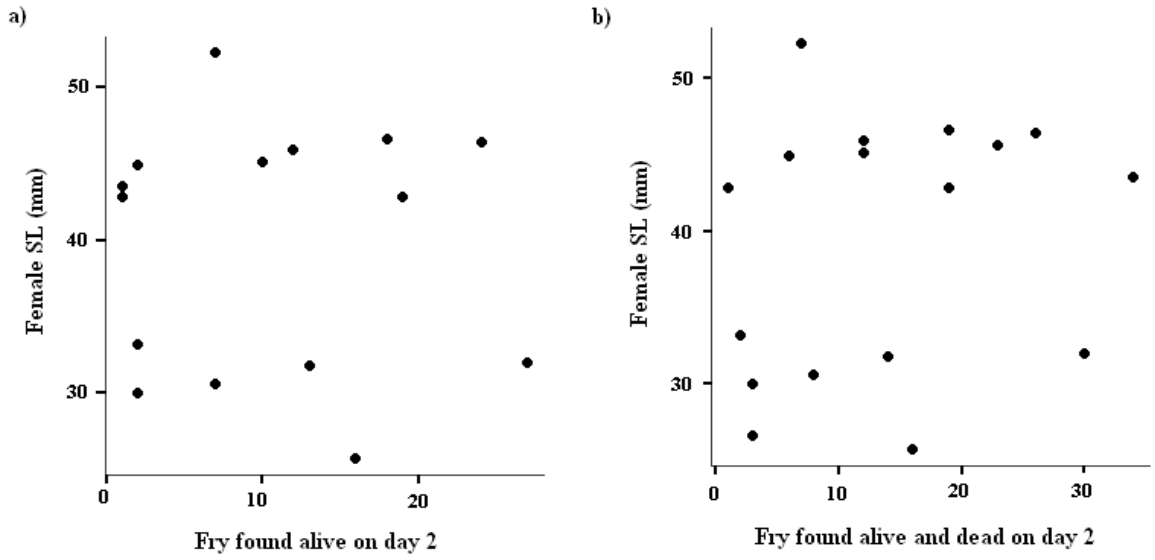

Figure 7-1. Graphs showing no relationship between female SL (mm) and a) the number of fry found alive on day 2 (P = 0.91), or b) the number of fry found alive and dead on day 2 (P = 0.40).

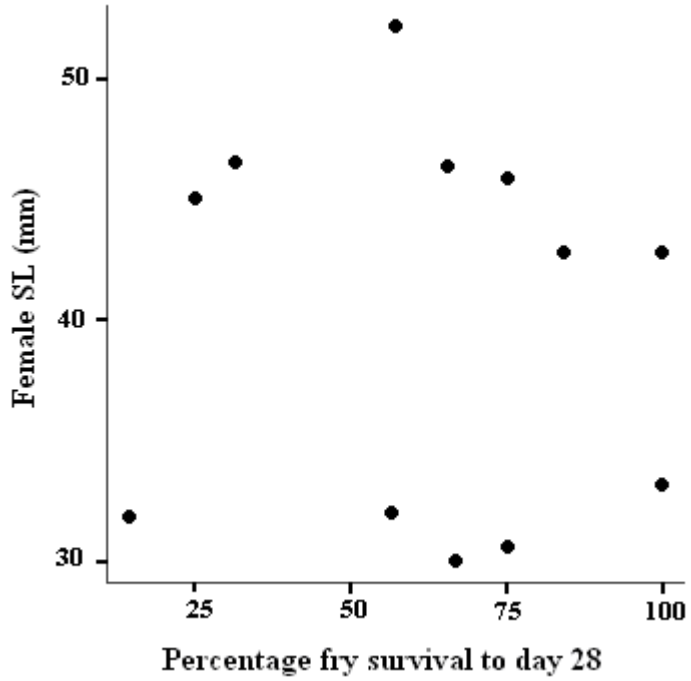

Figure 7-2. Graph showing no relationship between female SL (mm) and the percentage of fry surviving until day 28 (P = 0.82).

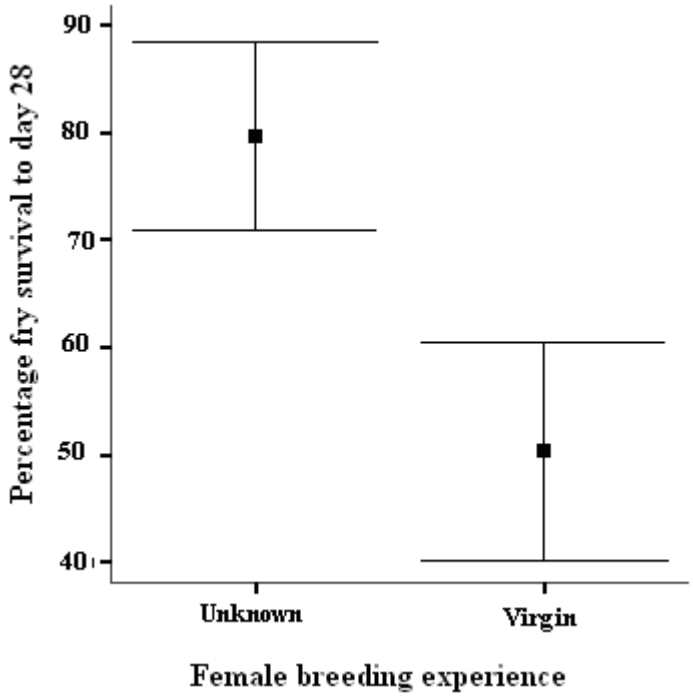

Figure 7-3. Graphs showing a non-significant trend for virgin females to have reduced fry survival compared to females of unknown breeding experience (P = 0.07). Error bars show mean percentage fry survival to day 28 ± S.E.

Discussion

I found that larger female green swordtails, *X. hellerii*, did not give birth to more, better surviving or quicker growing fry, compared to smaller females. Therefore, in this population of green swordtails, female size does not appear to correlate positively with fecundity. This result is counterintuitive, as I found in chapter 5 that male green swordtails prefer larger over smaller females; hence, it should be expected that males obtain some fitness gain through being choosy (Andersson, 1994). As males and females of this species show no parental care (Basolo, 1990), it seems likely that an increase in female fecundity may be the only fitness benefit a male could obtain by choosing to breed with large over small females.

The small sample sizes obtained during the breeding, may explain why I found no relationship between female size and fecundity. Only 15 females out of 24 gave birth to live fry and only 12 of these females had fry that survived to day 28. Furthermore, although I tried to prevent cannibalism, by providing shelters for the fry, females still were found to cannibalise their own offspring. Of the 20 females that gave birth, three of them completely cannibalised their brood, and it is possible that other females, unbeknown to me, also ate some of their broods before they were removed from them on day 2. This could have had severe effects on the results obtained, particularly if females differ in their propensity to cannibalise. Further, due to the availability of sexually mature females in my population, one of the breeding rounds used virgin females, whilst the other used non-virgin females. Due to the small sample sizes, these could not be analysed separately for looking at breeding success between the large and small females. However, analysis did show that, although breeding experience did not influence the initial number of fry that females gave birth to, the females of unknown breeding experience had a non-significant trend to have increased fry survival, over the virgin females. Further, more experienced breeders in several species have been found to have increased breeding success over less experienced pairs (Lunn et al., 1994; Nol & Smith, 1987; Ollason & Dunnet, 1978). Hence, it seems likely that my results may have been limited or influenced by the females I had available to breed with and their differences in breeding experience.

In conclusion, I have found no evidence that breeding with larger over smaller females, brings fitness advantages to male *X. hellerii*. However, small sample sizes, cannibalism of fry and differences in experience of the breeders may have confounded results and suppressed any effect of female size on fecundity. Alternatively, there may not be any increased fecundity fitness benefits to males picking large females. Instead, large females may just be easier to detect than small females, and hence reduce the costs of searching for mates. Larger females are also generally older than small females, as they grow throughout life, so size demonstrates the ability to survive, a measure of fitness. Thus, males may choose large over small females, as they may indirectly produce fitter offspring with increased survival. This study highlights the need to investigate what fitness benefits may be influencing individuals to be choosy. Nevertheless, in this species, further study into the fecundity of large versus small females is required, controlling for female experience. This should allow researchers to disentangle if males are choosing larger females for fecundity benefits, or if other factors are influencing their mate choices.

Appendix II: Validation of swabs as a non-destructive and relatively non-invasive DNA sampling method in fish

Abstract

Non-destructive methods of collecting DNA from small fish species can be problematic, as fin clips can potentially affect behaviour or survivorship in the wild. Swabbing body mucus may provide a less invasive method of DNA collection. However, risk of contamination from other individuals in high density groups could give erroneous genotyping results. We compared multilocus microsatellite genotypes from the same individuals when collected at low and high density and compared this with fin clips. We found no differences between these categories, with a genotyping error rate of 0.42%, validating the use of body mucus swabbing for DNA collection in fish.

Introduction

Acquiring high quality DNA is important for researchers investigating areas such as population genetics, the genetic diversity of threatened or endangered species or mating systems (O'Brien, 1994; Parker et al., 1998; Snow & Parker, 1998). However, collecting DNA samples can often be invasive, or involve sacrificing the animal. Blood sampling, for example, is a common means to obtain DNA, but in small fish it is often impossible to obtain blood without sacrificing the animal. Further, at least under UK legislation, it requires the fish to be anaesthetised and researchers collecting samples need to be trained and licensed. An alternative, non-destructive, means to collect DNA in fish is to take a small fin clip. However, this alters an individual's phenotype, which could have effects either on that individual's behaviour, or the behaviour of others towards it. In behavioural experiments this could influence results. Furthermore, fin clipping under UK legislation also requires that the researcher collecting the clip is licensed to do so and involves anaesthetisation and handling of the fish which can cause stress (Morales et al., 1990; Pirhonen & Schreck, 2003).

Sampling may also be detrimental to the animal's fitness, for example toe clipping in amphibians has been found to decrease survival in some species (Clarke, 1972; McCarthy & Parris, 2004). Some studies also suggest that fin clipping may affect survival (Hansen, 1988; Mears & Hatch, 1976; Weber & Wahle, 1969), although this is likely to carry a minimal risk. Using less invasive methods of collecting DNA that should be less stressful and less likely to affect behaviour are therefore favourable.

Swabbing fish for DNA requires very little training, no anaesthetisation, and can be carried out with a minimum of handling time. Sterile swabs are also cheap (around £16 for 100 swabs). Buccal swabs of fish have previously been used for DNA collection (Campanella & Smalley, 2006), but require fish with a large enough mouth to swab. In contrast, swabbing an individual's body mucus could be carried out on fish of all sizes. However, for fish living in high density groups there is potentially a high risk of contamination with DNA from other individuals, as individuals in close proximity may rub against each other, causing cells to slough off onto each other. As many fish used in laboratories are kept in high density tanks to reduce space and running costs, being able to reliably obtain individual DNA samples via swabbing that are cheap, quick and easy would be advantageous. Lucentini et al (2006) found that storing body mucus from brown trout, *Salmo trutta,* and northern pike, *Esox lucius,* on FTA Cards (Whatman) gave high quality DNA but they did not evaluate how stocking density might have influenced results.

Here, we investigated the risk of contamination and the quality and quantity of DNA obtained from swabs of body mucus in the small African cichlid species, *Neolamprologus pulcher*. The fish used ranged in size from 43 – 75 mm standard length. We investigated the risk of contamination of DNA from body mucus swabs by comparing: 1) swabs taken from individuals living in groups in small tanks (3-19 individuals in a 50 litre tank); 2) swabs taken when living in pairs in large tanks (two individuals in a 150 litre tank); and 3) fin clips. DNA was extracted and used for multi-locus microsatellite genotyping, with the same 30 individuals genotyped across all three contexts to evaluate consistency of results. If the swabs were contaminated with other individuals DNA, we would expect to find

inconsistent alleles between the swabs and the fin clips. Further, we quantified and assessed the quality of DNA between samples from swabs and fin clips.

Methods

One at a time, fish were netted quickly out of their tank and held in the net. The fish was then swabbed by running a Barloworld Scientific sterile rayon tipped swab (Fisher Scientific, UK) six times down the length of the body of the fish, from the pectoral fins to the start of the caudal fin. To obtain fin clips, fish had to be anaesthetised. Fin clips were taken from the caudal fin of each fish (approx 5 mm of tissue). Swabs and fin clips were then stored in 100% alcohol at 4°C prior to DNA extraction. The swabs taken when individuals were living in groups were stored for eight months, whilst the swabs taken from individuals living in pairs and the fin clips were only stored for 3-4 days before DNA extraction. DNA from swabs was extracted using an extraction protocol for swabs provided in the QIAamp DNA Micro Handbook (QIAGEN), using QIAshredder spin columns (QIAGEN) and the DNeasy Blood and Tissue Kit (QIAGEN). DNA from fin clips was extracted using the DNeasy Blood and Tissue Kit (QIAGEN), using the manufacturer's instructions. Individuals were then genotyped at eight microsatellite loci (UNH106, NP773PT, UL12PT, UME003PT, TmoM11PT, TmoM13PT, NP007PT and TmoM27PT) (Lee & Kocher, 1996; Parker & Kornfield, 1996; Schliewen *et al.*, 2001; Zardoya *et al.*, 1996). Products were amplified by multiplex PCR, using the default reagent concentrations recommended by the Qiagen multiplex kit instruction manual (Qiagen Inc, Crawley, UK). Two multiplex PCR's, one at 53°C (for UNH106, UL12PT, UME003PT, TmoM11PT and TmoM13PT) and the other at 60°C (for NP773PT, NP007PT and TmoM27PT), were carried out under the following conditions: initial denaturation for 15 min at 95°C, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at either 53°C for 60 s or 60°C for 90 s and extension at 72°C for 90 s, followed by a final 10 min extension at 72°C. Multiplexed products (1:160 dilutions) were sequenced using automated genotyping on an ABI 3730 sequencer (by The Sequencing Service, University of Dundee, UK). Genotypes were read, corrected by eye and analyzed using GeneMapper version 4.0 (Applied Biosystems, Foster City, CA). All alleles were called blind to the individual's identification and whether a fin clip or swab had yielded the DNA. DNA from swabs and fin clips

were also run on 2% agarose gels to assess DNA quality. In addition, all DNA samples were quantified using a Nanodrop ND-1000 spectrophotometer (Labtech International), with each sample being run three times and a mean calculated.

Results & Discussion

One microsatellite (TmoM27PT) was homozygous across all individuals, all others were heterozygous (observed heterozygosity; UNH106 = 0.5, NP773PT = 0.87, UL12PT = 0.97, UME003PT = 0.93, TmoM11PT = 0.87, TmoM13PT = 0.97, NP007PT = 0.53 and TmoM27PT = 0). For the fin clips, in one individual 4 microsatellites did not amplify (UL12PT, UME003PT, TmoM11PT and TmoM13PT) and in another two individuals one microsatellite (NP773PT) did not amplify. For the swabs taken from individuals living in a high density group, one further individual had one microsatellite (NP773PT) that did not amplify.

In all but one of the individuals sampled, the genotypes across the three different DNA collections were identical. In this one individual, at two of the microsatellite loci (TmoM13PT and UME003PT) that were heterozygous, both genotypes were identical for the two swabs, but were different from the fin clip. For the genotypes at locus TmoM13PT, the swabs and the fin clip had one allele in common but the other allele differed in size by three base pairs. In the other locus, UME003PT there was allelic dropout, where the fin clip did not amplify the allele at all, whereas both of the swabs had. This gave an overall genotyping error of only 0.42% for 1426 comparisons, so swabbing body mucus is a reliable DNA collection method. Further, figure 1 shows some smear indicating DNA degradation. However, the degradation does not appear to differ widely between the two sampling methods. Analysis of DNA yield found that swabs yielded less DNA (mean = 21.2 ± 7.26 ng/µl) compared to fin clips (mean = 65.9 ± 15.02 ng/µl) (see Appendix 1). However, this yield was still more than sufficient for our analysis.

In conclusion, this study validates the use of swabbing body mucus for reliably collecting DNA from fish. There was no evidence of contamination from cells sloughing off between individuals kept in high density tanks. Furthermore, although DNA yield was lower from swabs than from fin clips, the DNA quality

was sufficient for further analysis. Swabbing body mucus provides a non-destructive, relatively non-intrusive method for collection of DNA in fish, particularly when species are small and/or where behavioural experiments may be affected by altering an individual's phenotype. Therefore, this method would be effective in laboratory studies in which individuals are kept in high density tanks, DNA has to be collected from many individuals, where individuals are small and/or time for collecting DNA is limited. Sampling via swabs, would also be useful in the field, particularly for conservation projects when other methods of DNA collection may impact individual survival.

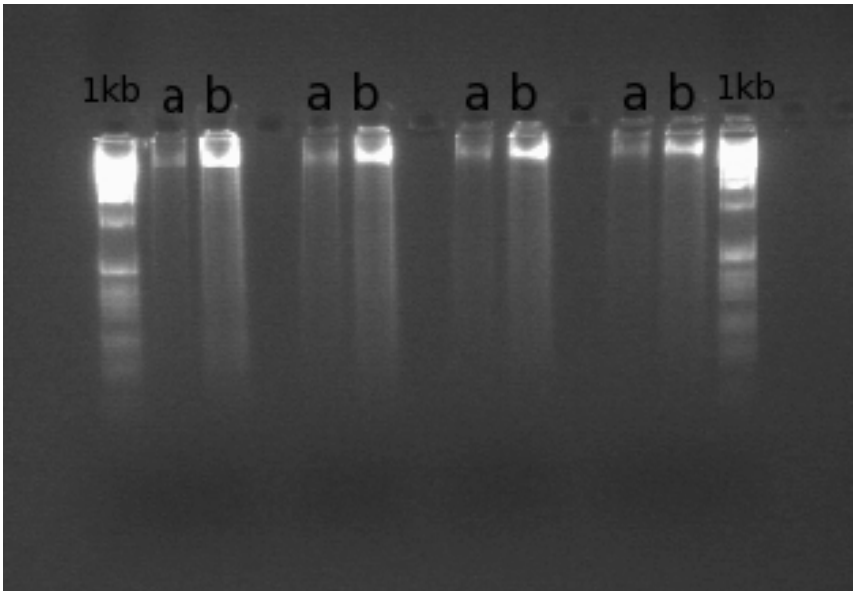

Figure 8-1. Agarose gel electrophoresis picture demonstrating the relative quantities and quality of DNA recovered from a) Swab samples, and b) Fin clips for four individuals. A 1kb DNA ladder is indicated.

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